

Allergens in the White and Yolk of Hen's Egg

A Study of IgE Binding by Egg Proteins

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Abstract. The radioallergosorbent test (RAST) was used to compare the IgE binding of egg white and yolk, and allergenic proteins were detected by immunoelectrotransfer ('Western blotting'). The main allergens were found in egg white, but for a large proportion of the egg-sensitive patients, yolk contained specific IgE-binding constituents. For blood sera from 36 patients, there was a positive correlation between the results of RAST for egg white and for yolk. Lysozyme was found to be an allergen for some patients. The effect of heating on the allergenicity of egg white was examined and the allergenicity of hen egg white was compared with that of a duck egg. The allergens in yolk were associated with each of the three yolk fractions, and several of the proteins in the low-density lipoprotein fraction bound IgE.

Introduction

Avian eggs, especially hen eggs, have long been known to contain protein allergens that invoke immediate (type I, IgE-mediated) reactions in susceptible patients, who are frequently young children [4, 5, 15, 18, 24, 29, 30]. According to most reports, these allergens are in egg white (i.e. albumen) although the yolk has sometimes been implicated as well [e.g. 29]. The allergenic properties of egg white have recently been studied by Langeland [21, 22], Langeland and Harbitz [23] and Hoffman [19]. Several potentially allergenic proteins from egg white have been studied in detail; e.g. ovomucoid [20] and conalbumin [23]. By contrast, egg yolk constituents have received little attention. Because of its possible importance for the study of food allergy, we are at present examining the allergenicity of the proteins of hen egg yolk by comparison with those of the white. This work follows a previous study of the antigenicity of egg-white proteins [26] and is a continuation of studies on the development of methods for identifying and separating egg yolk proteins [11-13]. As an initial step, we have used RAST to compare the effects of the total egg-white protein mixture with extracts of whole yolk using the sera from several patients allergic to egg. In addition, we have used Western blotting [14, 25] as a rapid

method for identifying allergenic egg proteins. As a consequence of the application of this method to egg white and yolk, we have re-examined the role of lysozyme as an allergen and we have made a provisional identification of allergens in one fraction of yolk.

Materials and Methods

Eggs and Egg Fractions

Eggs were from Australorp hens (*Gallus domesticus*) of known genetic strain maintained on a commercial mash diet at CSIRO Division of Animal Production, N.S.W. They were unfertilized and were used within a day or two of laying. After the yolk had been removed from most of the white using a separator, it was rolled on absorbent tissue paper to remove adhering white. The vitelline membrane was then pierced and the yolk squeezed out, avoiding contamination with the membrane or white.

For tests on unfractionated white, a dispersion in 0.1 M sodium bicarbonate or another salt solution was used. When necessary the relatively small fraction of insoluble material was centrifuged off. Egg lysozyme was isolated from a solution of the white by the method of Alderton and Fevold [1] and purified by ion-exchange chromatography on a column of carboxymethyl Sephadex C-50 as described by Back et al. [2].

Freeze-dried yolk extract was prepared as follows. The yolk was homogenized with an equal volume of 2 M sodium chloride and then centrifuged for 24 h at 4°C, 150,000 g (12 cm maximum radius). After centrifugation, the upper yellow lipoprotein layer was removed, homogenized with an equal volume of water, and dia-

Egg Allergens

lyzed for 48 h into three lots of 0.1 M ammonium bicarbonate at 2°C. The remaining yolk, containing the dissolved yolk granules and the soluble proteins, was dialyzed separately into ammonium bicarbonate solution. After dialysis the fractions were freeze-dried for 2 days. Finally the dried powders were well mixed.

The three fractions of egg yolk were isolated as follows. The yolk granules (23% by weight of the dry yolk [8]) were obtained by dispersing yolk in an equal volume of 0.16 M sodium chloride and centrifuging the mixture for 1 h at 100,000 g, 5°C. The granules sedimented as a firm pellet and were washed by dispersing in 0.16 M sodium chloride and recentrifuging. The supernatant solution was mixed with an equal volume of 4 M sodium chloride and centrifuged (100,000 g for 24 h, 5°C) to separate the low-density lipoprotein fraction (about 60% of the dry yolk) from the livetins fraction [11], i.e. the soluble yolk protein fraction, which was partly sedimented as a viscous pellet and partly in the supernatant solution. The centrifuge tube was sliced to give a clean separation of these fractions. No yolk was lost during this fractionation but, as discussed later, the possibility of contamination was not excluded. The livetins fraction contained almost no lipid. Lipid was removed from the other fractions with a mixture of chloroform and methanol in an adaptation of the method of Bligh and Dyer [6]. To give apoproteins that could be redissolved in detergent or other protein solvent, complete removal of lipid from the lipoprotein was essential [9, 10].

The Radioallergosorbent Test (RAST)

Filter-paper RAST discs were prepared as described by Barnett et al. [3], the amount of protein used being 100 µg/disc. The discs were then treated by the Phadebas procedure (Pharmacia AB, Bromma, Sweden).

RAST Inhibition Procedure

The method of Gleich et al. [16] as modified by Barnett et al. [3] was used in which the sera (50 µl) were incubated with 50-µl volumes of phosphate-buffered saline (0.05 M phosphate, 0.9% sodium chloride, pH 7.4) containing either 0, 5, 10, or 50 µg of protein. After 19 h, 50 µl of each was applied to an appropriate RAST disc containing the protein and incubated for 4 h. The discs were then subjected to the RAST procedure.

Skin Prick Test

The test solution consisted of 1 mg of protein in 1 ml of 1:1 water:glycerol. The allergenicity was gauged from the size of the reddened area when compared with non-allergic controls [7].

Immuno-Electrotransfer (Western Blotting) [14]

The proteins were separated by electrophoresis on polyacrylamide gel slabs (8 × 7.5 × 0.3 cm) using the modified method described by Sutton et al. [28] with the difference that the separating gel contained 10% acrylamide and the stacking gel 4% acrylamide.

Egg-white solution (20 mg/ml) was applied as a single streak (250 µl) across the slab. After electrophoresis a small strip of each slab was cut off and stained with Coomassie blue to check the separation of proteins. The rest of the slab was used for electrophoretic transfer to nitrocellulose membranes (0.1 µm pore size, Schleicher and Schull Ref. No. 402080). For this purpose a Hoefer Transfer Apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) was used at 100 V for 2 h with Towbin buffer that contained 20% methanol [28]. The membrane was initially blocked with phosphate-buffered saline that contained either 0.1% Tween 20 (for 1 h

at room temperature) or 0.25% gelatin (overnight at 4°C). The efficiency of transfer was checked by staining the polyacrylamide gel transferred although those with low electrophoretic mobility were transferred less completely. A strip of the nitrocellulose membrane was stained with amido black to reveal the positions of the protein bands and another strip was stained with fluorescamine to reveal the position of ovomucoid, which did not stain with amido black because of its high proportion of carbohydrate. Bands corresponding to allergens were detected by autoradiography or enzymically after incubating thin strips of the nitrocellulose membrane with patient's serum and Phadebas anti-IgE as described by Sutton et al. [28]. The enzyme method was used for figure 5 and 8. Although the protein bands were easy to detect on the nitrocellulose membranes, they were difficult to photograph. For figure 6 the appearance of the bands has therefore been indicated diagrammatically.

Egg-yolk proteins were examined by the same procedure with the difference that a 7.5% gel was used for electrophoresis.

Results

Tests on Egg White and Yolk before Separation of Fractions

Figure 1 shows a comparison of the results of RASTs on sera from 16 patients known to be clinically allergic to egg, and figure 2 summarizes the relationship between yolk and white RASTs for 36 patients including those used for figure 1. For the data in figure 2 the correlation coefficient was 0.690 ($p < 0.001$), which is highly significant. Few of the non-allergic patients tested gave RAST values of more than 3%.

The effect of heat on the allergenicity of egg white is illustrated in figure 3. The white was heated separately after dilution. According to these results, heating significantly decreased the allergenicity for less than half the patients. For the others, there was no difference or there was even an increase.

Figure 4 shows the results of a preliminary test designed to explore the cross-reactivity of egg white from different avian species by comparing hen with duck eggs. Results were similar for both species in about half the cases tested, suggesting a high degree of cross-reactivity, although the possibility of separate sensitization could not be excluded.

Tests on Egg-white Proteins; the Allergenicity of Lysozyme

Figure 5 gives an example of the application of Western blotting to the proteins of egg white using sera from 2 patients with high RAST values. The separated proteins were identified, where possible, by us-

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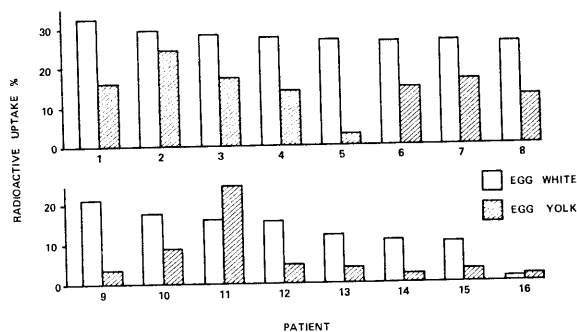


Fig. 1. A comparison of RASTs for egg-sensitive patients tested with hen egg white and yolk. For egg white (blank columns) a solution of the white in 0.1 M sodium bicarbonate was used. For the yolk (hatched columns) freeze-dried whole yolk was dispersed in 0.1 M sodium bicarbonate. Individual patients are represented by numbers on the abscissa. Some of the numbers in figure 3, 4 and 7 also refer to these patients.

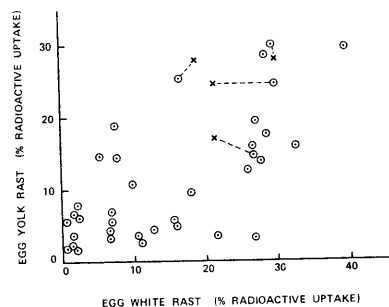


Fig. 2. Relationship between RASTs for hen egg white and yolk. In all, sera from 36 egg-sensitive patients were tested. As a measure of reproducibility, sera from 6 patients were measured twice at different times. Four of the duplicates are indicated by crosses with dashed lines joining the original values.

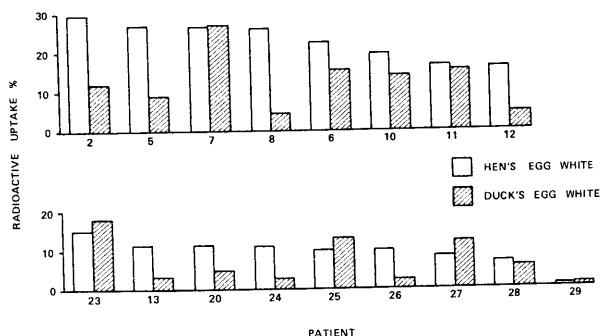


Fig. 4. RAST values used to determine the apparent cross-reactivity between egg white from hen and duck eggs. Sera from egg-sensitive patients were used. Hatched columns refer to duck egg white and the others to hen egg white.

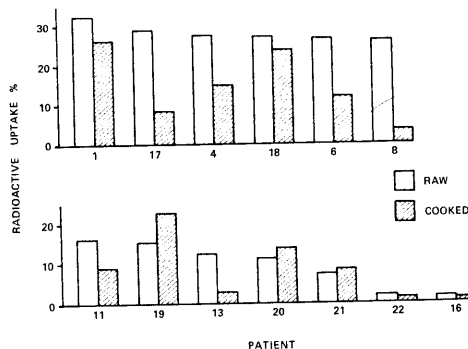


Fig. 3. Comparison of RASTs for raw and cooked white from hen eggs. The cooked samples (hatched columns) were prepared by diluting the white tenfold with water and heating at 90°C for 15 min.

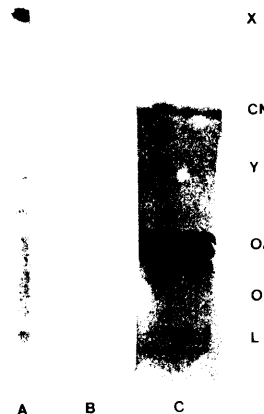


Fig. 5. Results of Western blotting, i.e. immuno-electrotransfer, on proteins of hen egg white. A, B Thin strips of the nitrocellulose membrane tested with sera from 2 egg-sensitive patients (see Methods). C Side strip of the same membrane, stained with amido black. CN = Conalbumin; OA = ovalbumin; L = lysozyme; O = position of ovomucoid (not revealed by amido black); X and Y are unidentified. An arrowhead marks the position of the top of the original gel. Electrophoresis was from top to bottom.

ing standards. Some of the allergens in figure 5 have not yet been identified. 10 patients were tested in this way and the results are summarized in figure 6. They show considerable variability. Of particular interest is the observation that lysozyme gave a positive result for all patients tested.

Figure 7 gives the results of RASTs with purified lysozyme for clinically egg-sensitive and non-allergic patients and includes their response to whole egg white. Table I gives other data for lysozyme, including the results of RAST inhibition and skin-prick tests which are in essential agreement with the results of the RASTs. It is of interest that 1 patient (No. 11, table I) gave a strong positive reaction to lysozyme but showed no clinical symptoms.

Tests on Fractions of Egg Yolk

Table II shows the results of RASTs on the three main protein fractions of egg yolk: granules, livetins, and low-density lipoprotein, compared with a dispersion of whole yolk. For these tests sera from 4 patients known to be highly RAST positive to yolk were used. The fractions were comparatively invariant and similar to the whole.

Figure 8 shows the results of Western blotting of the proteins from one fraction of egg yolk (the low-

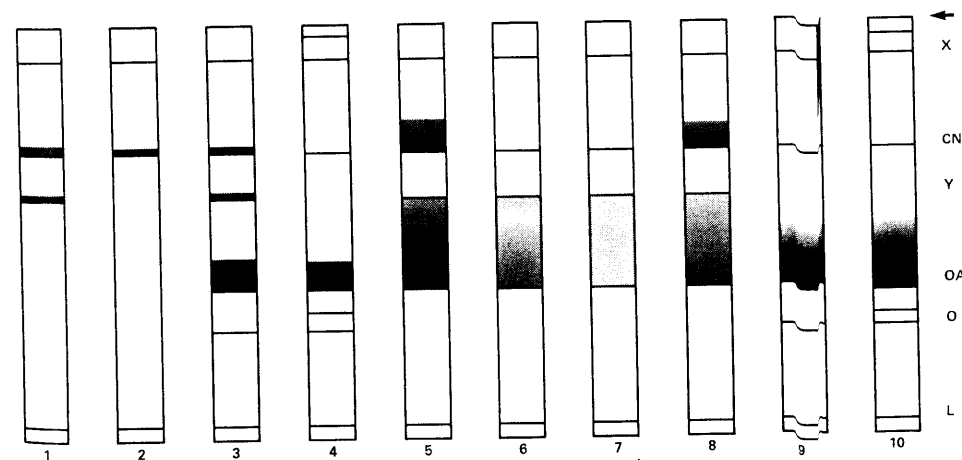


Fig. 6. Comparison of results for Western blotting for egg-white proteins using the sera from several egg-sensitive patients, represented by numbers 1-10. The numbers do not refer to the patients in figure 1. Each strip was copied from nitrocellulose membranes such as those described in figure 5, which also describes the lettering of the bands.

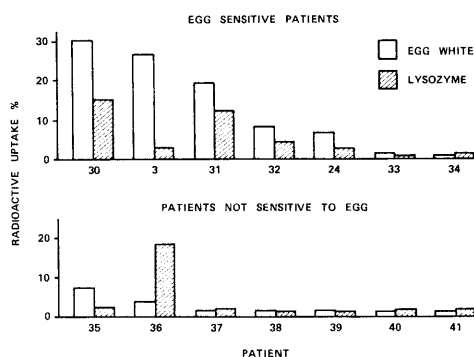


Fig. 7. Comparison of results for RAST for lysozyme with those for egg white. Patients sensitive to egg (upper group) and insensitive to egg (lower group) were used. The hatched columns refer to lysozyme and the others to whole egg white.

Table I. Results of RAST, RAST inhibition, and skin-prick tests on patients sensitive to egg and those with no history of egg sensitivity: a comparison of egg lysozyme with egg white

Patient ¹	Age years	Sex	Symptoms on eating egg	RAST, % of ¹²⁵ I uptake		RAST inhibition ²	Skin prick test
				egg white	lysozyme		
<i>Egg-sensitive patients</i>							
1	2	F	vomiting	30.5	15.1	70.2	+
2	7	F	facial oedema	27.2	3.1	0	+
3	7	F	facial oedema	26.5	14.0	nd	+
4	0.5	M	eczema	19.4	12.3	83.8	+
5	17	M	convulsion	8.4	8.3	66.1	±
6	7	M		7.2	3.1	0	+
7	34	F	eczema	2.3	2.6	nd	+
8	child	M	asthma, nausea	1.7	0.8	0	—
9	21	F	hives, vomiting	0.8	0.7	0	+
<i>Egg-insensitive patients</i>							
10	38	M	no reaction	7.5	2.6	0	—
11	6	M	no reaction	3.8	18.3	88.0	±
12	19	M	no reaction	1.7	1.5	0	—
13	10	M	no reaction	1.7	1.9	8.6	—
14	9	M	no reaction	1.6	1.4	nd	—
15	17	F	no reaction	1.5	0.9	nd	—
16	19	F	no reaction	1.5	2.0	nd	—

¹ These numbers do not correspond to those in figure 1-7 or in table II and III.

² Percentage inhibition of lysozyme discs by 50 µg of lysozyme (see Methods). Very low values could not be assessed quantitatively. These are designated 0; nd = not determined.

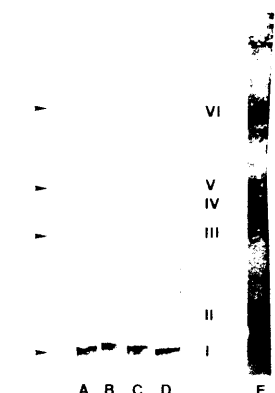


Fig. 8. Results of Western blotting on lipid-free proteins from the low-density lipoprotein of hen egg yolk. The membranes A-D refer to allergens in sera of 4 yolk-sensitive patients. Membrane E shows the positions of proteins revealed by staining with amido black. Roman numerals refer to the proteins, apovitellenins I-VI [9, 11]. Allergens are indicated by arrowheads on the left. Electrophoresis was from top to bottom.

Table II. Results of RAST on three fractions of hen egg yolk tested with sera from 4 highly yolk-sensitive patients

Patient	Whole yolk	RAST, radioactivity, % uptake		
		lipoprotein	livetins	granules
1	32.0	30.5	29.5	30.6
2	34.5	32.3	35.4	30.7
3	17.2	25.3	21.8	19.3
4	27.2	29.0	19.1	31.5

Table III. Results of RAST inhibition on sera from 6 patients highly allergic to egg¹

Patient	Egg-white discs inhibited by egg yolk, % inhibition ²	Egg-yolk discs inhibited by egg white, % inhibition ²
1	62.5	5.4
2	23.2	25.8
3	37.1	10.2
4	29.3	12.0
5	66.1	9.5
6	7.4	8.2

¹ Inhibition of discs by coincident extracts, i.e. white by white, yolk by yolk, under the same conditions, produced average inhibitions of over 70% for white and 65% for yolk using these sera.

² Percentage reduction in radioactive uptake following addition of 50 µg of inhibitor before incubation of disc.

density lipoprotein) using sera from the patients listed in table II. The dark lower bands correspond in mobility to a protein of very low molecular weight, apovitellenin I [9, 10, 12, 13]. In addition, there were at least three other bands corresponding to apovitellenins III, V, and VI [12].

Cross-Reactivity of Egg White and Egg Yolk

Table III provides evidence that for 6 egg-sensitive patients, there was cross-reactivity between the white and yolk of hen eggs.

Discussion

By applying RAST and Western blotting to sera from patients known to be allergic to hen eggs, we have compared the total allergenicity of egg white and yolk and have explored some of the individual allergens. As suggested by Sutton et al. [28], Western blotting has proved to be especially useful. This technique

has shown that all the abundant egg-white proteins bind IgE to some extent (fig. 6). The wide variations amongst patients help to explain why different laboratories have favored different proteins for the principal allergen. In making such comparisons, the differences in the proportions by weight of the egg-white proteins should be borne in mind; namely, ovalbumin 54%, conalbumin 12%, ovomucoid 11%, ovomucin 1.5%, lysozyme 3.5%, plus 18% not properly identified. Of particular interest is the observation that lysozyme is an allergen according to its IgE-binding capacity. This observation has led us to a more detailed study using RAST, RAST inhibition, and skin-prick tests on purified lysozyme (fig. 7; table I). From these tests it is clear that for some of the patients tested lysozyme is indeed a potent allergen. For others it is possibly an irritant rather than an allergen. This result contrasts with the conclusions of Langeland [21, 22] and Langeland and Harbitz [23] from a study using other methods. The reasons for this difference require further investigation but they may be an example of population differences; alternatively, the specificities of the different techniques may need re-examination.

Two allergenic proteins that have not yet been identified were detected by Western blotting (fig. 6) in egg white. One of these is of very high molecular weight and may represent a fraction of ovomucin, which is responsible for the high viscosity of egg white [27].

An important aspect of our work is the indication that egg yolk also binds IgE for patients allergic to egg (fig. 1). In fact, in our tests there was a significant positive correlation between the allergenicities of egg white and yolk (fig. 2). Egg white bound more IgE, as expected from previous studies. Yolk is a more complicated system than egg white, and includes a large proportion of lipid, so identification of the allergens poses special problems and is still in progress. RAST results (table II) indicated that equal weights of the three main fractions of yolk (low-density lipoprotein, livetins, granules) all bind IgE to approximately the same extent. This result is puzzling but it may be an indication that the methods used did not give uncontaminated fractions. It is known, for example, that a low-density lipoprotein is part of the granules [8]. More conclusive results would be expected from Western blotting, which has so far been applied to the major fraction, the low-density lipoprotein (fig. 8). This material evidently contains several protein allergens.

Although there are no obvious similarities between proteins of egg white (which are synthesized in the oviduct) and yolk (which are synthesized largely in the liver), evidence has recently been presented [17] that there are homologies in the DNA sequences of some of their genes, thus implying evolution from a common ancestral gene. It is therefore possible that the yolk and white proteins have sequence homologies involving the IgE-binding sites. Our evidence for cross-reactivity (table III) would be in line with this suggestion. Only a distant relationship would be expected because the two groups of protein are now distinctly different and patients respond to them differently (fig. 1). Nevertheless, any such relationship would help to simplify the study of the molecular features necessary for egg allergy in particular and food allergy in general.

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