

A Clinical and Immunological Study of Allergy to Hen's Egg White

V. Purification and Identification of a Major Allergen (Antigen 22) in Hen's Egg White

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Antigen 22 (ag-22) in hen's egg white, previously shown to be one of the major allergens in the egg white, was partially purified by combining biochemical separation techniques and quantitative immunoelectrophoretic methods. The molecular weight of ag-22 was found to be approximately 78,000 using analytical ultracentrifugation and pI was determined to be 6.1, which is appropriate with the values of ovotransferrin. It was concluded that ag-22 is identical with ovotransferrin. The ability of ovotransferrin to react in the human IgE-system was demonstrated *in vivo* and *in vitro*, by means of skin prick tests and crossed radioimmuno-electrophoresis.

Key words: food allergy; hen's egg white; major allergens; ovotransferrin; quantitative immunoelectrophoresis.

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Previously, 68 single sera from egg allergic individuals were analysed with respect to specific IgE-antibodies to individual proteins in the hen's egg white. This was done by means of crossed radioimmuno-electrophoresis (CRIE) (9). In this study there were found three major allergens in the egg white. The major allergens were shown to be ovalbumin, ovomucoid and antigen 22 (ag-22), denoted in order of most frequent causation of reactions in CRIE. Thirty-six of the 68 allergic sera gave rise to radiostaining of precipitate No. 22, the reactions being strong (CRIE-classes A-D) in 20 of these cases.

It is not known which protein ag-22 represents among the approximately 40 proteins present in egg white (17). The aim of the present study was to purify ag-22 in order to perform an identification of this protein.

MATERIAL AND METHODS

Reference antigen solution and antisera

A hen's egg white reference antigen solution was prepared by stirring fresh egg white gently for 4 h in an equal volume of 0.15 mol/l NaCl, followed by centrifugation (2500 rpm, 30 min) as previously described in detail (8).

A polyvalent rabbit antiserum against hen's egg white, shown to contain precipitating antibodies to 24 antigens in hen's egg white (8) was used. The production of this reference antiserum is described elsewhere (8).

A monospecific antiserum against ag-22 was prepared by immunizing four rabbits with immunoprecipitated ag-22, obtained from CIE-slides. The immunoprecipitated ag-22 was re-

patient with atopic dermatitis and total IgG > 4000 U/ml.

Quantitative immunoelectrophoresis

CIE was performed on glass slides ($5 \times 7 \text{ cm}^2$) with equipment and reagents as described in detail previously (8). CIE with intermediate gel (CIEWIG) and crossed line immunoelectrophoresis (CLIE) were performed according to Løwenstein (12). In these experiments, cathodically moving proteins were excluded from the patterns. In the CLIE-experiments an additional intermediate gel was used ($1.5 \times 5 \times 0.13 \text{ cm}^2$) to separate the ag-22 containing gel from the antibody-containing gel. Otherwise the conditions in CIEWIG and CLIE were as described for the CIE-reference pattern.

Rocket immunoelectrophoresis was performed at 2 V cm^{-1} for 19 h, according to Weeke (18), with equipment and reagents as described for CIE. In order to estimate the amounts of ag-22 in various fractions of the egg white, samples were run against the monospecific anti-ag-22-antiserum (1.4 mm thick gel, containing $14 \mu\text{l}$ antiserum/ cm^2). Rocket immunoelectrophoresis was also used to determine the amount of ovalbumin in skin prick test solutions b and c. In these experiments the monospecific anti-ovalbumin antiserum was used (1.4 mm thick gel, containing $7 \mu\text{l}$ antiserum/ cm^2).

Following electrophoresis, the slides were washed, pressed and dried before staining with Coomassie Brilliant Blue (12).

CRIE was performed as previously described (9), incubating four CIE-slides (ag-22 against hen's egg white reference antiserum) with single sera from egg allergic patients and controls ($400 \mu\text{l}$ serum in a total volume of 7 ml). Subsequently, the slides were incubated with ^{125}I -anti IgE (Pharmacia, Uppsala, Sweden) (250,000 cpm in a total volume of 7 ml), and finally placed face-down on an X-ray film for autoradiography for 7 days.

Skin prick test (SPT) was performed preferentially on the volar surface of the forearm (10). The results were recorded according to

the Scandinavian allergy reference system, using histamine HCl 1 mg/ml as the positive control (+++) and physiological saline as the negative control (-) (2). The allergen solutions used in SPT were as follows: a) the hen's egg white reference antigen solution, undiluted, b) ag-22 dissolved in 0.15 mol/l NaCl (5 mg/ml, determined according to Lowry (13)), c) ovalbumin, dialysed against physiological saline, freeze-dried and dissolved in 0.15 mol/l NaCl to 0.05 mg/ml protein concentration. SPT with b and c was performed in 15 individuals with suspected egg allergy based on history and positive SPT (++ or more) with the hen's egg white reference antigen solution. Twenty-five subsequent patients with atopic dermatitis and egg tolerance and negative SPT with allergen solution a, were tested with allergen solution b as a control.

Isolation of antigen 22

Fresh egg white was stirred gently for 4 h at $+4^\circ\text{C}$ in an equal volume of 0.15 mol/l NaCl and centrifuged at 10,000 rpm for 30 min. (JA-14 rotor, Beckman J21 centrifuge). The supernatant was stored frozen at -40°C . A 50 ml sample was thawed at $+4^\circ\text{C}$ and dialysed for 24 h against $2 \times 1 \text{ l}$ 0.1 mol/l $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ -buffer, pH 4.9. The sample was centrifuged at 10,000 rpm for 30 min at $+4^\circ\text{C}$ and the supernatant fractionated on a CM-Sepharose CL-6B (Pharmacia Fine Chemicals) ion exchange chromatography column ($5.0 \times 12.5 \text{ cm}^2$). The column was eluted stepwise with 0.1 mol/l $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ -buffers: pH 4.9, pH 6.0 and pH 6.7, respectively, and finally with 0.1 mol/l glycine/NaOH-buffer, pH 11.0 containing 1.0 mol/l Na_2SO_4 (16). The fraction containing antigen 22 (identified using rocket immunoelectrophoresis) was eluted with the buffer at pH 6.7. This fraction was dialysed against $2 \times 1 \text{ l}$ 0.15 mol/l NaCl for 24 h at $+4^\circ\text{C}$, and thereafter an equal amount of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ ($+4^\circ\text{C}$) was added (50% saturation). The solution was centrifuged at 10,000 rpm for 30 min, the sediment was discarded while the supernatant was saturated to 73% with $(\text{NH}_4)_2\text{SO}_4$.

The sediment was isolated by centrifugation dissolved in 0.15 mol/l NaCl and shown to contain antigen 22. The sample was dialysed against 2×1 l 0.15 mol/l NaCl for 24 h at $+4^{\circ}\text{C}$, concentrated on a PM10 Diaflo ultrafilter (Amicon) and finally gel filtrated on a Sephacryl S-300 column ($2.6 \times 82 \text{ cm}^2$) (Pharmacia Fine Chemicals) eluted with 0.15 mol/l NaCl. The column was calibrated with solutions of ribonuclease, ovalbumin, albumin, aldolase, catalase, ferritin and thyroglobulin.

Electrophoresis

Sodium dodecyl sulfate (SDS) – electrophoresis was carried out as described by Porzio & Pearson (15) with modifications as described earlier

(6). Aliquots (50 μl) containing approx. 40 μg of antigen 22 were layered on the gel. The method was calibrated using the molecular weight standards from Bio Rad.

Isoelectric focusing

The isoelectric focusing was performed in a 110 ml Ampholine electrofocusing column (LKB) in a pH 3.5–10 ampholyte gradient. 3 ml ampholyte solution (LKB Cat. 1809-001) and approx. 15 mg of purified antigen 22 were added to the entire volume of the column and the experiment was run at 2.5 W for 44 h. $\text{OD}_{280\text{nm}}$ and pH were determined in 2 ml fractions after elution of the column.

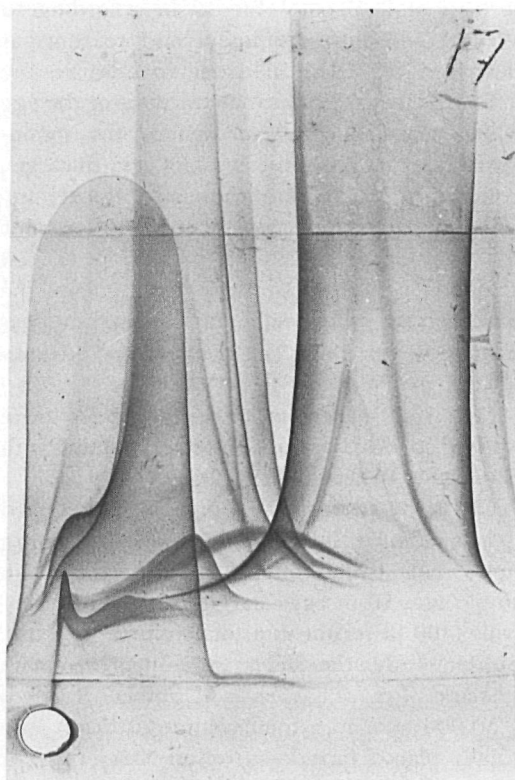
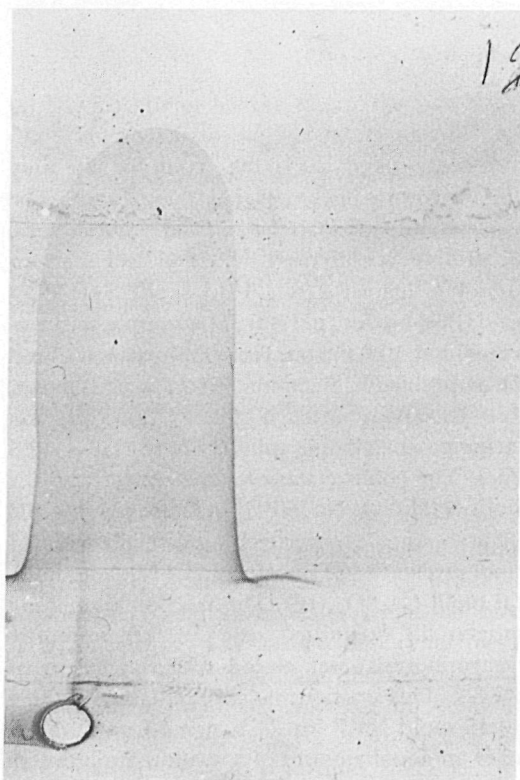


Fig. 2. Examination of antiserum against ag-22.

a) CIE with the hen's egg white reference antigen solution against the antiserum against ag-22. Cathodically moving proteins were excluded, otherwise conditions as described for the CIE reference pattern fro hen's egg white.

b) CIEWIG with the antiserum against ag-22 added to the intermediate gel. Cathodically moving proteins were excluded, otherwise conditions as described for the CIE-reference pattern for hen's egg white.

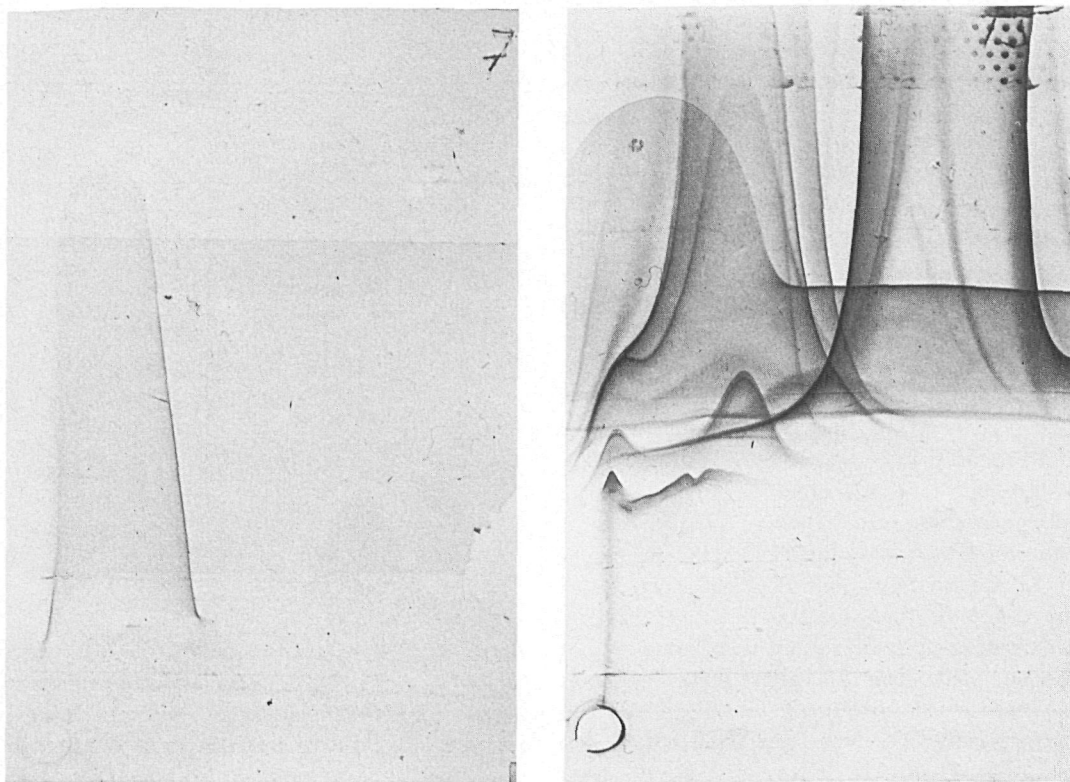


Fig. 3. Examination of the purified ag-22 preparation.

a) CIE with ag-22 preparation as antigen against the hen's egg white reference antiserum. Cathodically moving proteins were excluded, otherwise conditions as described for the CIE-reference pattern for hen's egg white.

b) CLIE with the ag-22 preparation added to the intermediate gel next to the first dimensional gel. Cathodically moving proteins were excluded, and an additional intermediate gel was used. Otherwise, conditions as described for the CIE-reference pattern for hen's egg white.

Analytical ultracentrifugation. The purified ag-22 preparation was analysed using a Beckman, Mod E ultracentrifuge with double beam photoelectric scanner at 280 nm for optical registration. The concentration of the protein sample was 0.5 mg/ml, dissolved in 0.1 mol/l phosphate buffer, pH 7.0. The partial specific volume was taken to be 0.72 ml/g. The molecular weight was determined at sedimentation equilibrium in two experiments, at 8,000 and 12,000 rpm, respectively.

The affinity of antigen 22 for ferrous ions

The affinity of ag-22 for ferrous ions was investigated according to Donovan & Ross (5).

Ferrous ammonium sulfate ($\text{Fe}[\text{NH}_4]_2[\text{SO}_4]_2 \cdot 6 \text{H}_2\text{O}$) was dissolved in distilled water (0.02 mol/l). 10 μl aliquots of this solution were added to a 3 ml sample of ag-22 (5.0 mg/ml) in 0.15 mol/l NaCl. Absorbance at 470 nm (indicating the degree of binding of ferric ions to the protein) was determined after 30 s, 1 min, 2 min, 3 min, 5 min and 10 min.

RESULTS

The CIE-reference pattern for hen's egg white is shown in Fig. 1. This pattern has been discussed in detail elsewhere (8).

CIE with the hen's egg white reference antigen solution against the anti-ag-22 antiserum,

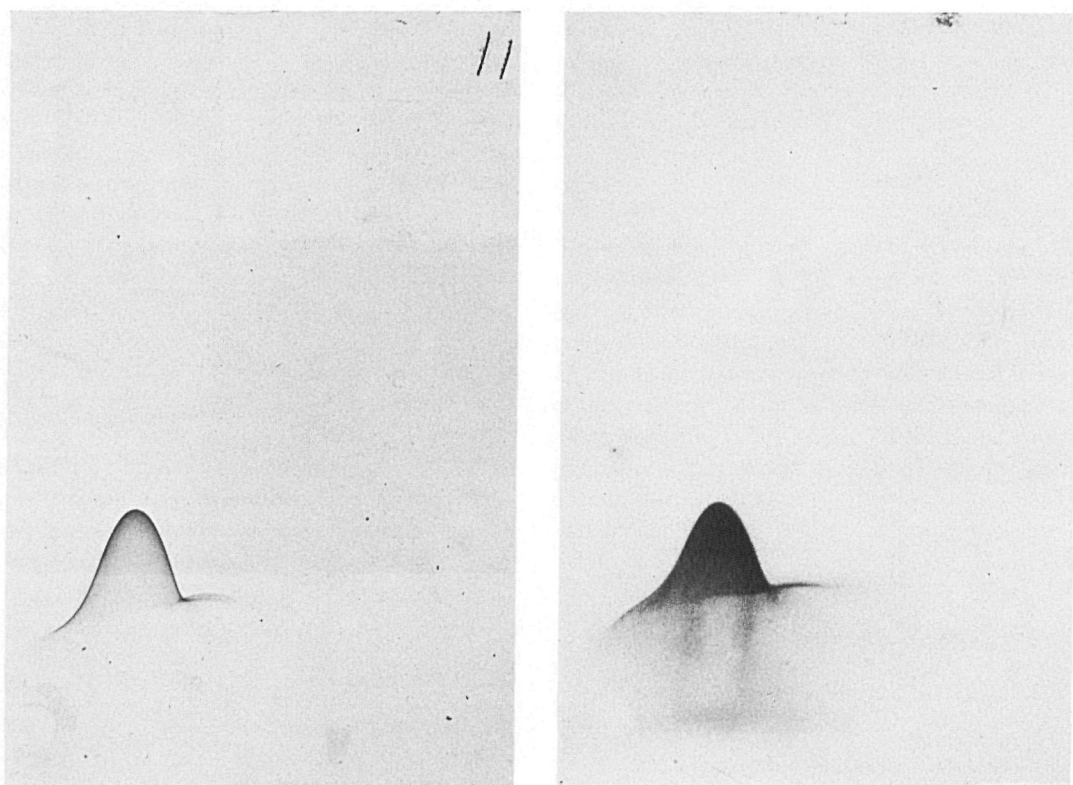


Fig. 4. CIE/CRIE experiments with ag-22.

- a) CIE with the ag-22 preparation against the hen's egg white reference antiserum stained with Coomassie Brilliant Blue.
- b) CRIE X-ray film with radio-staining corresponding to the CIE-precipitate of ag-22.

demonstrated one precipitate only (Fig. 2a). CIEWIG with anti-ag-22 antiserum added to the intermediate gel, demonstrated the presence of precipitating antibodies against ag-22 in this antiserum (Fig. 2b).

Fresh egg white was fractionated following a procedure in three steps (as described), to give a sample of antigen 22 of high purity, as demonstrated by SDS-electrophoresis and gel-filtration (not shown). From the two latter methods the molecular weight of ag-22 was estimated to be 61,000 and 63,000, respectively. By means of analytical ultracentrifugation the molecular weight of ag-22 was, however, determined to be 78,000 and 77,400 at 8,000 and 12,000 rpm, respectively. The small difference between the two values obtained indicates a high

degree of homogeneity of the ag-22 preparation.

Isoelectric focusing of the protein (2 experiments) gave a mean $pI = 6.1$.

CIE with the purified ag-22 preparation against the hen's egg white reference antiserum, demonstrated one precipitate only, localized in the area where ag-22 was expected to be (Fig. 3a). CLIE-experiments demonstrated an immunological identity reaction between the purified ag-22 preparation and precipitate No. 22 in the CIE-reference pattern for hen's egg white (Fig. 3b). Precipitate No. 13 was constantly found to disappear in these experiments. Furthermore, a slight elevation of precipitate No. 6 (corresponding to ovalbumin) could be seen in some experiments, adding high amounts (ap-

Table 1
Skin prick test results

	Hen's egg white ref. antigen solution	Ag-22 5 mg/ml	Ovalbumin 0.05 mg/ml
K.H.	>++++	++	+
T.G.	>++++	++++	++
H.P.	>++++	+++	++
V.S.	>++++	++	+
G.R.	++++	+++	-
N.L.	++++	+	-
S.H.	>++++	+++	-
K.S.	+++	++	+
K.H.	++++	+++	+
C.D.	++++	++	-
O.B.	++++	+	-
D.S.	+++	-	-
G.Ø.	+++	-	-
A.S.	+++	-	-
T.S.	++++	-	-

prox. 100 µg protein) of the ag-22 preparation to the intermediate gel.

CRIE experiments with sera from egg allergic patients all demonstrated strong radio-staining of the precipitate obtained in CIE with the ag-22 preparation (Fig. 4). Eleven out of 15 patients with positive reactions in SPT with the hen's egg white reference solution (a), were positive in SPT with the solution of ag-22 (b) as well (Table 1). The controls (25 patients) were all negative in SPT with the ag-22-solution. By means of CIE, the allergen solutions a and b were found to be equally concentrated in ag-22. Only six of the patients were positive in SPT with the dilute solution of ovalbumin (c). The positive reactions with the ag-22-solution can therefore not result from only a minor contamination of ovalbumin in this solution. In rocket immunoelectrophoresis the concentration of ovalbumin in solution b was shown to be only approx. half of the concentration of ovalbumin in solution c, which means that the contamination only represents 0.5% of the protein in the ag-22-solution (b).

DISCUSSION

The purification of ag-22 was performed by combining quantitative immunoelectrophoretic

methods and biochemical separation techniques. The monospecific anti-ag-22 antiserum was crucial in these experiments, making this work much easier. The monospecificity of the antiserum was documented in CIE and its specificity to ag-22 in CIEWIG.

The immunization of rabbits with ag-22, present as immunoprecipitates in gels, proved to be successful. Ag-22 seems to be a potent antigen, as the four rabbits all responded well with production of precipitating antibodies to this immunization, although the amount of ag-22 injected on each occasion was estimated to be less than 1 µg.

CIE and CRIE experiments with the purified ag-22 preparation demonstrated a high degree of purity of this protein. This was confirmed by means of SDS-electrophoresis, gel filtration, isoelectric focusing and ultracentrifugation.

In CLIE, there was constantly found an interaction between the purified ag-22 preparation and ag-13, being an intermediate allergen in hen's egg white (9). Retrospective examination of the hen's egg white CIE-reference pattern revealed a partial identity-reaction between these two proteins. This is probably the explanation for the disappearing of ag-13 in CLIE, adding ag-22 to the intermediate gel, although ag-13 itself could not be detected in the purified ag-22-preparation.

The common antigenic determinants, being the basis for the partial immunological identity between ag-22 and ag-13, are probably not dominating in the human IgE-system. This is because simultaneous radio-staining of ag-22 and ag-13 has been an inconstant finding in previous CRIE-experiments with hen's egg white (9).

The characterization of the purified protein in CIE and CLIE, and the CRIE-experiments clearly demonstrate that the purified protein really is ag-22. A comparison of the molecular weight determined by analytical ultracentrifugation (78,000) and the isoelectric point (pI = 6.1) of ag-22 found in this study, with those of ovotransferrin found in the literature (14), strongly suggests that ag-22 is identical with ovotransferrin. Further evidence for this conclusion is the ratio between the amount of ag-22

obtained and the amount of egg white used in the separation, indicating that ag-22 is one of the quantitatively dominating proteins in hen's egg white. The identity of ag-22 was finally verified by adding ferrous ammonium sulfate to the protein solution in accordance with Donovan & Ross (5) and measuring a dramatic increase in absorbance at 470 nm resulting from the characteristic ability of ovotransferrin to bind iron atoms.

The extraordinary low values for molecular weight resulting from SDS-electrophoresis and gel filtration may result from the tightly bound tertiary structure of this protein, stabilized with 14 disulfide bonds (19).

Previous literature on allergens in hen's egg white has paid little attention to ovotransferrin (3). Bleumink & Young did not find this protein to be of importance when investigating the skin reactivity of various fractions of the egg white (4). An explanation for this may be that their opportunities to identify and characterize the test preparations were limited, in the absence of today's immunoelectrophoretic techniques. In the present study the ability of ovotransferrin to react in the human IgE-system has been documented, *in vivo* and *in vitro*, by means of SPT and CRIE, respectively.

Ovotransferrin has a molecular weight which is higher than most other allergens characterized in the human IgE-system (1). The size of this protein is large with respect to its ability to penetrate the intestinal mucosa. It remains to be known whether fragments of the protein assumed to be formed during digestion, could act as allergens in egg allergic individuals.

The three major allergens in hen's egg white, ovalbumin, ovomucoid and ovotransferrin, have all been thoroughly studied from a biochemical point of view (17).

Which properties of these proteins make them the most important allergens in hen's egg white is so far an open question. It should be emphasized that the major allergens are also the three most quantitatively dominant proteins in the egg white, together constituting approx. 75% of the total protein amount in hen's egg white (17).

This fact, probably not coincidental, may at least be one important reason why these particular proteins give rise to the highest IgE titre among egg allergic patients.

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