

Characterization of Four Major Allergens of Hen Egg-White by IEF/SDS-PAGE Combined with Electrophoretic Transfer and IgE-Immunoautoradiography

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Abstract. The antigenic and allergenic epitope maps of the hen egg-white ovalbumin, ovomucoid, ovotransferrin and lysozyme were obtained using one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis and two-dimensional sodium dodecyl sulfate gel electrophoresis following isoelectric focusing. The isoelectric points of egg-white proteins were assigned by the isoelectric focusing technique. Two-dimensional electrophoresis following isoelectric focusing provided further information regarding epitope mapping. Furthermore, electrophoretic transfer of the proteins to nitrocellulose and subsequent immunoautoradiography, clearly demonstrated the allergenicity of these proteins. An important benefit of these methods was confirming that lysozyme bound strongly to IgE in all the human sera from egg-allergic individuals and that lysozyme, in addition to ovalbumin, ovomucoid and ovotransferrin, was one of the major allergens of hen egg-white.

Introduction

Hen egg-white is an essential ingredient in human food. The proteins of hen egg-white are common causes of hypersensitivity symptoms among atopic individuals. Egg is ubiquitously used as food and hence not easy to eliminate from the diet. The biochemical composition of hen egg-white has been studied intensively. Five major components, namely ovalbumin (OA), ovomucoid (OM), ovotransferrin (OTr), ovomucin (Omc) and lysozyme (Lsz), represent about 80% of the total protein amount. The remaining 20% are composed of at least 35 different minor proteins, some of them familiar, but many are not yet characterized [22]. The complete primary structure of hen OA has been elucidated [20], and the antigenic structure of many proteins has been determined [2, 9, 28]. The decapeptide, amino acid residues No. 1-10 of hen's egg OA, has been synthesized by our group, and the result suggested that the NH₂-terminal segment encompassed an Ig-binding haptenic epitope [10]. Recently, we have also synthesized seven other OA peptides [13]. Among the different hen egg-white proteins,

only 3 were demonstrated to be major allergens: OA, OTr and OM [3-5, 8, 16-18].

Crossed immunoelectrophoresis (CIE) and crossed radioimmunoelectrophoresis (CRIE) are analytical methods widely used in detecting proteins in allergic extracts [14, 23, 24].

Studies on the antigens and allergens of hen egg-white by CIE and CRIE has previously been performed by Langeland [16, 17] in 1982 and Langeland and Harbitz [18] in 1983. On the other hand, isoelectric focusing (IEF) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) combined with immunoautoradiography (IAR) give better resolution than the traditional CIE and CRIE, and represent good alternatives for epitope mapping.

The aim of the present paper is to derive antigenic and allergenic epitope maps of OA, OM, OTr and Lsz, and compare them by using one-dimensional (1-D) electrophoresis, IEF, and the combined two-dimensional (2-D) technique, and furthermore, to investigate the allergenic reactivities of gel-separated OA, OM, OTr and Lsz by specific IgE binding to these proteins after being transferred to nitrocellulose (NC) membranes.

Materials and Methods

Hen Egg-White Proteins

OA, from chicken egg, type VII, A-7641; OM, from chicken egg-white, trypsin inhibitor, type III-0, T-2011; OTr, from chicken egg-white, conalbumin type 1, C-0755, and Lsz, from egg-white, L-4631 were purchased from Sigma Chemical Co., USA. Crude hen egg-white extract was prepared as described earlier [9].

Human Sera

The individual human sera and serum pool were collected from 10 patients with RAST class 2-4 towards egg. All patients were allergic to egg or egg and fish. Equal aliquots of sera were used for the pool. None of the patients had previously undergone immunotherapy, and the blood samples were donated for research purpose. Serum samples were discarded after being used for the incubation procedures described later.

One-Dimensional Electrophoresis

Gel electrophoresis was done by 4% stacking, and 12% separating gel [15]. The material to be tested was handled according to the BioRad Protean™ II Slab Cell Instruction Manual (BioRad Chemical Division, Richmond, Calif.). In each well, 0.33 ng of the different protein samples were analyzed. The Coomassie brilliant blue, R 250 (CBB; Sigma) staining procedure was used to visualize the protein bands [27].

Two-Dimensional Electrophoresis

Fingerprints of the proteins were obtained by horizontal IEF at pH gradient 4-6 and 3.5-10.5. Aliquots of 15 and 30 μ l (1-3 μ g/ μ l) were used, and the equipment of LKB, Bromma, Sweden was utilized [29].

Electrophoresis in the second dimension was done in 10% SDS-PAGE. A phosphate electrode buffer system (0.1 M, pH 7.1) was used, and the gels were prerun for 30 min with a preset constant current of 150 mA. The IEF strips were equilibrated in a phosphate sample solution (0.01 M, pH 7.1) with 1% SDS and 1% mercaptoethanol before electrophoresis. The electrophoresis was performed with a preset constant current of 215 mA. Molecular weight (Mr) marker was added to the gel to ensure a proper protein migration. Horizontal LKB 2117 multiphor electrophoresis unit (LKB, Bromma, Sweden) was used in both the IEF and 2-D SDS-PAGE analyses.

Electrophoretic Transfer and Immunoautoradiography

Electrophoretic transfer of the separated proteins was performed as described in previous publications [6, 12, 21]. The gels were transferred overnight; IEF gels in 1% acetic acid at 20 V/cm² and SDS gel in glycine-methanol buffer (192 mM 20% methanol, 25 mM Tris-HCl, pH 8.3 at 7.7 V/cm²). The IAR technique used was a modification of procedure outlined by Burnette [6]. The NC blots from IEF and SDS gels were washed in 70 mM NaCl-phosphate buffer (pH 7.2; PBS), and blocked with 10% bovine serum albumin (Sigma) in PBS. The blots were washed and incubated with the serum pool, followed by washing and incubation with ¹²⁵I-anti-IgE (Phadebas RAST isotope, Pharmacia, Sweden), with a radioactivity of more than 70,000 cpm/ml. The NC immunoblots were exposed at -80°C using Kodak AR 5 film. The exposed films were developed in a Kodak X-Omat RP automated processor for 90 s at 33°C [11].

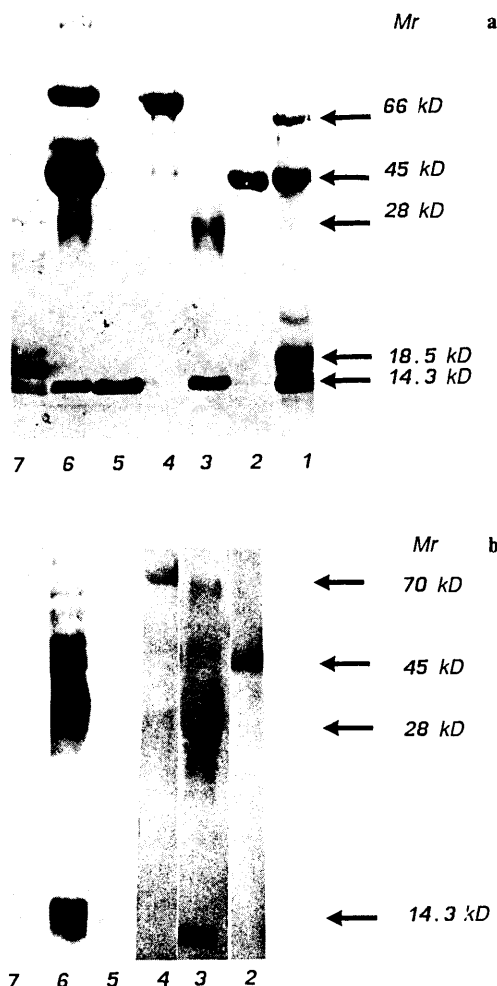


Fig. 1. 1-D SDS-PAGE and autoradiographic illustration of hen egg-white proteins. **a** The CBB-stained 1-D SDS gel analysis of: lane 1, Mr marker (bovine serum albumin 66 kD, OA 45 kD, β -lactoglobulin 18.5 kD, Lsz 14.3 kD); lane 2, OA; lane 3, OM; lane 4, OTr; lane 5, Lsz; lane 6, crude hen egg-white extract; lane 7, a control lane of birch crude extract. **b** Immunoautoradiographic binding of patient serum-specific IgE against hen egg proteins. Lane 2, OA; lane 3, OM; lane 4, OTr; lane 5, Lsz; lane 6, egg-white crude extract; lane 7, Birch crude extract (control lane).

Results

One-Dimensional Electrophoresis

The Mr distribution of OA, OM, OTr, Lsz and crude hen egg-white was obtained by vertical 12% 1-D SDS-PAGE. As visualized in figure 1a, OA, OM, OTr and Lsz (lanes 2-5) gave distinct CBB-stained protein bands corresponding to Mr of 45, 27-28, 66-70 and

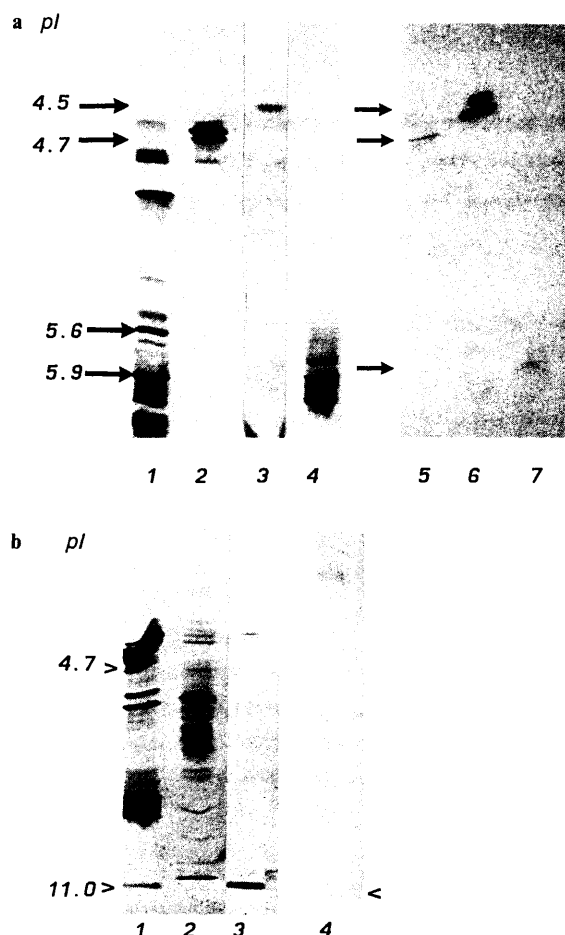


Fig. 2. IEF mapping and immunoradiographic binding of patient serum-specific IgE of the proteins in hen egg-white extract. **a** IEF using pH gradient 4–6. Lane 1, pI-marker proteins; lane 2, OA (15 μ g); lane 3, OM (45 μ g); lane 4, OTr (30 μ g). The corresponding autoradiographic signals are visualized in lane 5 (OA), lane 6 (OM) and lane 7 (OTr). **b** IEF using pH gradient 3.5–10.5. lane 1, Hen egg-white crude extract; lane 2, birch crude extract (control); lane 3, Lsz. The autoradiographic signal of Lsz is pictured in lane 4.

14.3 kD, respectively. In lane 1 a Mr marker is shown. A crude hen egg-white reference extract (lane 6) consisted of two heavily stained protein bands of Mr 66–70 and 45 kD, as well as a distinct 14.3-kD band. Furthermore, three minor bands were visible at 77, 50 and 27–28 kD. The autoradiographic picture of patient specific IgE binding (fig. 1b) demonstrated strong signals at 45 kD (OA, lane 2), at 27–28 kD (OM, lane 3), at 66–70 kD (OTr, lane 4) and finally at 14.3 kD (Lsz, lane 5). Lane 6 gave the IgE-binding pattern of hen egg-white extract. A control, lane 7

(fig. 1a, b), using birch pollen crude extract [23] and human egg serum pool, was set up to demonstrate the specificity of the run. In the 1-D gel the commercial egg-white proteins showed a varying degree of purity which will be discussed later.

Isoelectric Focusing

OA, OM and OTr were resolved by IEF in a pH 4–6 gradient into series of 4, 2 and approximately 10 CBB-stained bands, respectively (fig. 2a, lanes 2–4). Lane 1 shows the pI-marker proteins (LKB). Both the stained OA, OM and OTr bands (lanes 2–4) and the corresponding immunoradiographic signals (lanes 5–7) were located between pH 4.63–5 (OA, lanes 2 and 5), pH 4.4–4.5 (OM, lanes 3 and 6) and pH 5.6–6.2 (OTr, lanes 4 and 7). The two strongest autoradiographic signals were detected between pH 4.4 and 4.5 (OM, lane 6).

Crude hen egg-white extract and Lsz were analyzed in a pH 3.5–10.5 gradient IEF gel (fig. 2b). Crude hen egg-white (lane 1) could be resolved into 25–30 bands; 11 in the pH region 4.5–5.

Lsz (lane 3) gave 2 bands: a strongly stained band at pH 11 and a faint band at pH 4.6 (probably OVA contaminant). The immunoblot picture of Lsz (lane 4) gave one weak signal at pH 11, while using birch crude extract as a control (stained in lane 2), the birch crude extract gave no binding of IgE from egg-allergic serum.

Two-Dimensional Electrophoresis

The egg-white proteins analyzed by 2-D electrophoresis did verify the location and number of protein bands obtained in the IEF picture. As these proteins are already characterized in figures 1 and 2, only the 2-D fingerprints of OM and Lsz are emphasized: OM for being nondetectable by regular staining procedures [2, 8, 16], and for reporting Lsz as an additional major egg-white allergen. The 2-D fingerprint of OM, shown in figure 3a, consisted of two protein spots corresponding to a Mr of about 28 kD located between pH 4.4 and 4.6. Another CBB-stained protein, 14.3 kD, was also visible in the same pH region. IgE binding of OM, demonstrated in figure 3b, was detected as one strong signal showing the greatest intensity at Mr 27–28 kD and one faint signal in the same Mr region. The signal was detected between pH 4.4 and 4.6 in the IEF gel.

Figure 4a shows the 2-D peptide map of Lsz as well as one IEF strip. The 2-D run gave a strongly stained

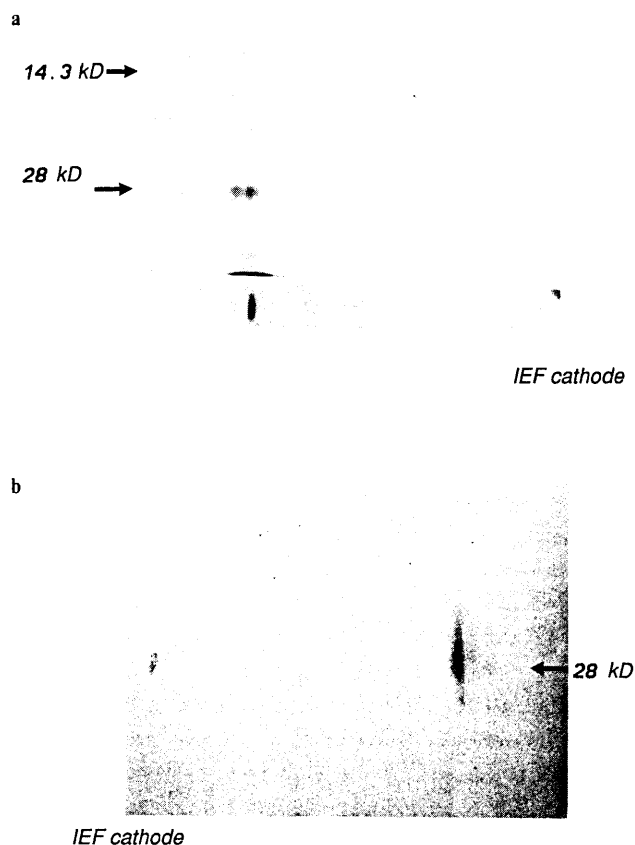


Fig. 3. 2-D electrophoresis and autoradiography of OM. **a** The orientation of OM proteins separated by IEF on a 2-D SDS-polyacrylamide gel. **b** Visualize the IgE-binding ability of 2-D gel-separated OM proteins. The blot is a mirror image of the 2-D gel pictured in figure 3a. The position of the IEF cathode is also indicated.

spot in the Mr 14.3-kD region, and one spot at 35 kD, both corresponding to pH 11 in the IEF gel. In the pH-4.6 region, one spot was visible corresponding to Mr 45 kD. Lsz did bind IgE from the human egg serum pool, visualized by strong signals corresponding to the stained spots in the 2-D gel. The strong IgE-binding ability of Lsz is seen in figure 4b.

Discussion

The aim of the present communication was to demonstrate the number of hen egg-white proteins binding specific IgE from individual sera and serum pools of egg-allergic patients. We used available egg-white

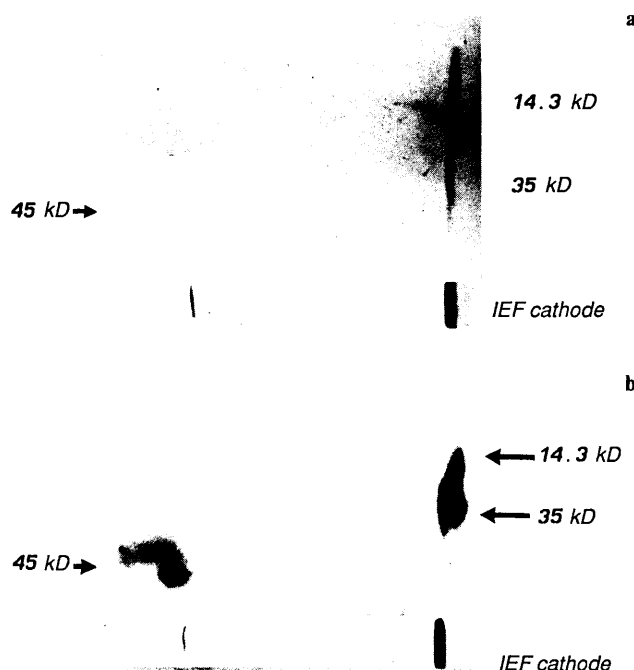


Fig. 4. 2-D electrophoresis and autoradiography of Lsz. **a** Upper, 2-D electrophoresis; lower, IEF, pH 3.5–10.5 gradient gel. Lsz was separated according to the protein's isoelectric points in the first dimension, and according to their molecular weight in the second dimension. **b** Visualize the autoradiographic signals obtained after the 2-D electrophoresis-separated Lsz protein was blotted into NC and incubated with patient egg serum and ^{125} I-anti-IgE.

proteins which had limited degrees of purity. The contaminating proteins were easy to detect, as were other allergens from hen egg-white, as indicated by their IgE binding. The heterogeneity detected when analyzing these proteins was mainly due to the sensitivity of the IAR method.

The quantitatively most dominating allergen in hen egg-white, OA, seemed relatively homogenous when analyzed either by IEF or 1-D SDS-PAGE. In the 1-D gel OA was only contaminated by a faint 70-kD protein band. This could either be a technical artefact, since it did not bind IgE in the same blot, or traces of OTr. OA analyzed by IEF gave no CBB staining of OTr proteins. However, in some of the 2-D gels, traces of CBB-stained proteins moving along the

Mr range from 66 to 28 kD at pH 5.6–6.4 were visible (picture not shown). The corresponding protein band traces were visible as faint signals in the IAR pattern of OA after 48-hour film exposure. Prolonged exposure gave stronger signals (plates not shown).

OM is also an important major allergen demonstrated in previous studies [5, 6, 14, 16]. The protein used in these experiments was contaminated by OTr, OA and a heavily stained Lsz band visualized in 1-D SDS-PAGE. The 4 protein bands gave IgE binding in the immunoblot picture, especially the OM- and Lsz-protein bands which showed strong IgE binding. The lack of detection of OM by regular staining methods in agarose gel electrophoresis, SDS-PAGE, IEF or CIE has been reported previously [2, 8, 16]. This may be due to differences in the resolving power of the methods and experimental conditions used. One important fact is that OM acts as a weak immunogen in rabbits [8], and the animals may not give rise to antibodies that can be demonstrated in CIE. In the present paper there was no difficulty in detecting OM following the described 1-D SDS-PAGE procedure. In IEF gels stained with CBB, OM was only visible when the protein concentration applied on the gel was not less than 45 µg. OM (45 µg) gave weaker staining than OA (15 µg) and OTr (30 µg) in the IEF gel, probably because OM has a high carbohydrate content, approximately 20% [22].

When the IAR method was applied, the IEF gel gave three signals of which the strongest corresponded to OM. The other signals were related to OA and OTr. The heavy signal of OM was probably caused by the high protein concentration (45 µg) applied, compared to OA (15 µg) and OTr (30 µg). The IAR picture of OM in the 2-D gel verified, after prolonged (96h) film exposure, two autoradiographic signals corresponding to the two protein spots visualized in the IEF gel.

Another important major allergen of hen egg-white, OTr [17, 28] showed one contaminating protein band, probably OA in the CBB-stained 1-D picture. The immunoblot picture gave strong IgE binding to OTr and faint autoradiographic signals corresponding to OA and the 27- 28-kD band (OM), not visible in the CBB-stained gel. The 2-D gels of OTr showed poor separation between the different protein bands visible in the IEF gel. After 96-hour exposure, the 2-D blot gave one strong signal, probably the same protein band that gave IgE binding in the IEF blot. The role of Lsz as an allergen has been debated [4]. Miller

and Campbell [19] concluded that Lsz was the most important allergen in egg-white. This was not confirmed by later studies [4, 25], which regarded Lsz as an irritant giving nonspecific skin reactions. Lange-land [17] showed one immunologically specific precipitate (No. 24) in CIE; however, he could not detect any specific IgE antibodies against this protein in any of the patient sera analyzed. Recently, Lsz was reported to be an important allergen for egg-allergic patients, particularly some incidences of severe type-I allergic reactions to chicken egg Lsz in drugs [1, 7, 26].

As an allergen, Lsz was, due to its high pI and low Mr, difficult to demonstrate by conventional methods [17], in spite of being very well characterized [2]. Our results clearly showed that IgE from all the egg-allergic patients' sera and pool, bound to Lsz included in the crude extract and OM preparation as visualized by 1-D SDS gels and IAR (fig. 1b). Furthermore, by 1-D IAR analysis, Lsz strongly bound IgE, as did other protein contaminants such as OA, OM and OTr. Two different egg serum pools and three different individual sera from egg-allergic patients were particularly tested in the 1-D IAR system. IgE in all the sera tested bound to variable degrees to the Lsz band immobilized into NC. Identical egg serum pools and individual sera were used in the 2-D IAR system with comparable results. A birch crude preparation control, showed no detectable IgE binding [23].

In conclusion, the present analysis of 4 hen egg-white proteins have, for the first time, simultaneously visualized all the 4 proteins as clinically important allergens.

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