

Purification of egg-white allergens

Ebbehøj K, Dahl AM, Frøkiær H, Nørgaard A, Poulsen LK, Barkholt V.
Purification of egg-white allergens.
Allergy 1995; 50: 133–141. © Munksgaard 1995.

K. Ebbehøj¹, A. M. Dahl¹, H. Frøkiær¹,
A. Nørgaard², L. K. Poulsen²,
V. Barkholt¹

¹ Department of Biochemistry and Nutrition,
Centre for Food Research, Technical University
of Denmark, Lyngby; ² Laboratory of Medical
Allergology, Allergy Unit, National
University Hospital, Copenhagen, Denmark

Purification procedures for the four egg-white proteins ovomucoid, ovotransferrin, ovalbumin, and lysozyme are presented with reference to mechanistic studies at epitope levels of allergic reactions to these proteins. The applied procedures resulted in four preparations containing less than 0.1% contaminating proteins each. The purified protein preparations were characterized by SDS-PAGE and by crossed immunoelectrophoresis with polyclonal antibodies raised against an egg-white extract or the purified proteins. The necessity of these well-characterized proteins in studies on allergic reactions was shown by testing human sera in immunoblots of lysozyme, and by immunoblots of ovomucoid probing with antibodies against the proteins.

Key words: egg protein immunology; food allergens; food allergy; food hypersensitivity; hen's egg white; lysozyme; ovalbumin; ovomucoid; ovotransferrin.

Kirsten Ebbehøj
Department of Biochemistry and Nutrition
Building 224
Technical University of Denmark
DK-2800 Lyngby
Denmark

Accepted for publication 25 July 1994

Hen's egg is one of the foods most frequently reported to elicit allergic reactions (7, 8, 25, 26, 28, 30). Most studies have focused on egg-white proteins, which are reported to elicit allergic reactions more frequently than egg yolk (2, 4, 21). Egg white is a complex mixture of more than 20 proteins, among which the four proteins ovomucoid, ovotransferrin, ovalbumin, and lysozyme have been claimed to be the main allergens (2, 15, 20, 22). Detailed studies of the immunologic response require highly purified proteins and antibodies against these proteins, as shown in recent studies of IgE binding of the individual proteins (1). It has been shown that egg white generally retains much ability to bind specific IgE even upon harsh heat treatment (29). Because widely different heat stability and resistance to both chemical and enzymatic cleavage are found in different egg-white proteins, the purity becomes even more important when investigating how such treatments as heating or fragmentation of proteins affect allergens and thereby allergic reactions. A minor protein impurity resistant to cleavage could lead to erroneous interpretation of studies of easily degradable proteins. The commercial protein preparations have been shown to contain large amounts of cross-contaminating proteins (varying from less than 1% to more than 5%; unpublished data); thus, purification of these preparations is required. Similar impurities were also found by Hoffman (15), who, in addition, purified the proteins, but without presenting data from the purification steps.

The aim of the present work was, through relatively simple purification procedures, to obtain highly purified protein preparations of the four main egg-white proteins ovomucoid, ovotransferrin, ovalbumin, and lysozyme. This is necessary for studies on the immune response at the protein level and at the epitope level, taking into consideration both T-cell and B-cell receptors. Moreover, for characterization of the purified proteins, antibodies against the purified proteins and egg-white extract were raised and characterized.

The aim of the present work was, through relatively simple purification procedures, to obtain highly purified protein preparations of the four main egg-white proteins ovomucoid, ovotransferrin, ovalbumin, and lysozyme. This is necessary for studies on the immune response at the protein level and at the epitope level, taking into consideration both T-cell and B-cell receptors. Moreover, for characterization of the purified proteins, antibodies against the purified proteins and egg-white extract were raised and characterized.

Material and methods

Reagents

Antiserum raised against Kunitz soybean trypsin inhibitor (α -KSTI pAb) (9) and monoclonal antibodies (mAbs) against ovomucoid (α -OM mAb 3E5) raised according to procedures described by Ebbehøj et al. (12) were used. The latter has been characterized as an IgM antibody that reacts with both native ovomucoid and reduced and alkylated ovomucoid. Alkaline phosphatase-conjugated swine antirabbit

immunoglobulins (D306) and rabbit antimouse immunoglobulins (D314) were purchased from Dako (Glostrup, Denmark). ^{125}I -labeled rabbit anti-human-IgE and M_r marker (80-1129-85) were obtained from Pharmacia (Uppsala, Sweden). Agarose (HSA, batch 6281) was purchased from Litex (Glostrup, Denmark), Tris (trizma-base, T1503) was purchased from Sigma Chemical Co. (St Louis, MO, USA), and, acrylamide (10675) was purchased from Serva Feinbiochemica (Heidelberg, Germany). The purity of these three chemicals has been found to be crucial for the respective methods.

Human sera

Sera were obtained from patients who had been characterized by double-blind, placebo-controlled food challenges, as previously described (26). For the present study, sera were selected on the basis of a positive response (class 3 +) when tested for specific IgE against egg-white extract (Phadebas RAST, Pharmacia) and positive IgE response to lysozyme (L-6876, Sigma Chemical Co) in dot-blot immunoassays. The IgE binding in these sera to individual egg-white proteins has previously been described (1).

Egg-white proteins

The preparations of ovomucoid (T-2011, lot 20H8170), ovotransferrin (C-0880, lot 104F8065), ovalbumin (A-7641, lot 43F8050), and lysozyme (L-6876, lot 39F8212) were purchased from Sigma Chemical Co. The preparations designated *crude* throughout this paper were of the highest obtainable degree of purity and hence very often used in studies of allergic reactions. Egg-white extract (EW) was prepared as described by Langeland (19).

Purification of proteins

Commercial preparations of the four proteins ovomucoid (OM), ovotransferrin (OT), ovalbumin (OA), and lysozyme (LY) were subjected to a two-step purification comprising ion-exchange chromatography done on fast performance liquid chromatography (FPLC) (Pharmacia, Uppsala, Sweden), followed by gel filtration.

Ovomucoid, ovalbumin, and ovotransferrin. Anionic-exchange chromatography was done on a Q-Sepharose FF column (XK16/20, Pharmacia) for OM and OA, whereas, for OT, a Mono Q HR 5/5 column (Pharmacia) was used. For all three proteins, 20 mM triethanolamine, pH 7.3, was used as eluent, and desorption was done by a salt gradient from 0 to 1 M NaCl, 20 mM triethanolamine, pH 7.3. OT was, before ion-exchange chromatography,

depleted of Fe^{3+} by extensive dialysis of the protein (20 mg/ml) dissolved in 50 mM EDTA, pH 4.5, against 50 mM EDTA, pH 4.5.

Lysozyme. LY was purified by cationic-exchange chromatography on a Mono S HR 5/5 (Pharmacia) with 20 mM Bicine-HCl, pH 8.7, as eluent. Desorption was done by a salt gradient from 0 to 1 M NaCl, 20 mM Bicine, pH 8.7.

Pooled fractions from each protein were freeze-dried, dissolved in 50 mM NH_4HCO_3 , pH 7.8, and purified by gel filtration in 50 mM NH_4HCO_3 , pH 7.8, on a Sephacryl S-200 HR column (XK16/100, Pharmacia).

Preparation of antisera

Polyclonal antibodies (pAb) were obtained by immunizing Black Russian rabbits. Each immunization consisted of a total of 1 ml emulsion of 200 μg protein in Freund's incomplete adjuvant given in four simultaneous injections. Antisera were raised against purified OM (α -OM pAb), purified OA (α -OA pAb), partially purified OT (α -OT pAb), purified LY (α -LY pAb), and egg-white extract (α -EW pAb), respectively. Immunizations were given at 5-week intervals, and blood was taken 8 days after each immunization. One serum against each protein was chosen on the basis of antibody titer evaluated by crossed immunoelectrophoresis (CIE). In a similar way, the purity of the pAbs against the specific proteins was determined by CIE against egg-white extract. The sera were aliquoted, stored at -20°C , and used without purification.

Crossed immunoelectrophoresis (CIE)

CIE was done on glass slides ($5 \times 10 \text{ cm}^2$) according to Clarke & Freeman (10), with equipment, as described by Weeke (32), using 1% agarose gel (w/v) in 73.5 mM Tris and 24 mM 5,5-diethylbarbituric acid, pH 8.6. The first and second dimensional electrophoresis procedures were run at 13°C , at 20 V/ cm^{-1} for 1 h and 2 V/ cm^{-1} for 16 h, respectively. OA was applied in amounts of 30 μg /well, OT in amounts of 40 μg /well, and OM and LY were applied in amounts of 50 μg /well, all in volumes of 5–20 μl . The second dimensional gels contained α -EW pAb 5 $\mu\text{l}/\text{cm}^2$, α -LY pAb 3 $\mu\text{l}/\text{cm}^2$, and α -OM pAb 3 $\mu\text{l}/\text{cm}^2$.

SDS-PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was done in 10% acrylamide gels, as described by Schägger & von Jagow (31), on a dual cooled vertical slab electrophoresis

Purification of egg-white allergens

unit (SE 600, Hoefer Scientific, CA, USA). Protein samples dissolved in Tris/SDS sample buffer containing 40 mM dithiothreitol were boiled for 5 min before electrophoresis at 24 mA constant current for 19 h at 15°C. Protein samples of 5 and 1 µg, respectively, were loaded onto the gels. The gels were stained with 0.025% Serva Blue G (31) or were silver stained (5).

Immunoblots of SDS-PAGE

Preparative SDS-PAGE of crude and purified proteins, respectively, were electrotransferred to nitrocellulose by semidry blotting (18) modified with methanol in the blotting buffer (23). The membranes were soaked in 2% Tween 20, 50 mM Tris, and 150 mM NaCl, pH 10.3, to prevent nonspecific binding. Then they were cut into lanes of 5 mm, dried, and stored refrigerated or processed immediately.

Specific IgE against EW-proteins was determined in immunoblot analysis, as described by Jarolim et al. (16), with serum from patients RAST-positive for one or more egg-white proteins. Strips of LY blots were soaked in 50 mM Tris, 150 mM NaCl, 0.5% (w/v) Tween 20, and 0.01% NaN₃, pH 7.4 (TBS/Tween 20), with addition of 3% (w/v) skim-milk powder to prevent nonspecific binding. Specific IgE was then detected by incubation with the human serum diluted 1:10 in TBS/Tween 20, followed by incubation with ¹²⁵I-labeled rabbit antihuman IgE (100 000 cpm/strip) and, finally, development by autoradiography at -80°C with Amersham Hyper-MP film and intensifying screens.

Reactions with rabbit or mouse antibodies were done as described by Ramlau (27), the strips being incubated overnight with pAbs or mAbs diluted 1:100 in 1.5 ml incubation buffer (0.5% Tween 20, 50 mM Tris, and 150 mM NaCl, pH 10.3). The detector antibodies were alkaline phosphatase-conjugated swine antirabbit immunoglobulins for the pAbs and rabbit antimouse immunoglobulins for the murine α-OM mAb, respectively. The blots were developed with nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate as color substrate. From each gel, one strip including a *M_r* marker was stained with gold colloid solution (24).

Results

Characterization of the crude protein preparations

Testing the purity of the crude protein preparations revealed that they contained several components. Immunoblots with human sera showed IgE binding to several proteins different from LY, but present in very small amounts as contaminants in the LY preparation; only one serum (no. 3) showed binding to LY at *M_r* 14 400 (Fig. 1). When LY was purified

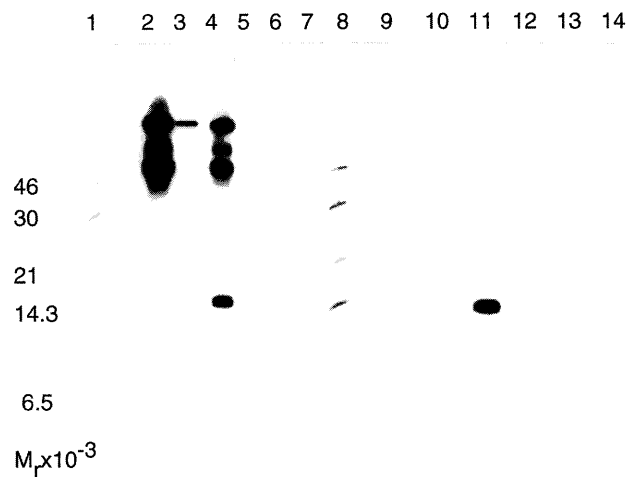


Fig. 1. Binding of human IgE to protein blots of LY. Lanes 2-7: crude LY; 9-14: purified LY. 1) and 8): *M_r*-marker; 2) and 9): serum no. 1; 3) and 10): serum no. 2; 4) and 11): serum no. 3; 5) and 12): serum no. 4; 6) and 13): serum no. 5; 7) and 14): serum no. 6.

(see below) and retested in immunoblot, the only IgE reaction remaining was from serum no. 3, although the sera used in the study were initially selected on the basis of positive IgE reactions in nonseparating dot-blot immunoassays.

Investigating the crude OM preparation by immunoblotting with pAb revealed the same pattern (Fig. 2). Immunodetection with pAb against the four individual proteins or α-EW pAb revealed several protein bands. Staining of the blot by colloid gold gave exactly the same protein bands seen from the α-EW pAb incubation of the blot. With α-OM pAb,

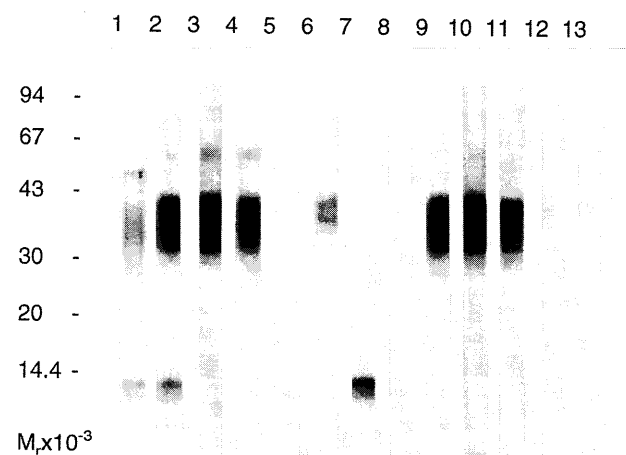


Fig. 2. Protein blots of crude OM probed with lane 1) gold colloid reagent; 2) α-EW pAb; 3) α-OM pAb; 4) α-OM mAb 3E5; 5) α-OT pAb; 6) α-OA pAb; 7) α-LY pAb; 8) α-KSTI pAb. Purified OM probed with 9) α-EW pAb; 10) α-OM pAb; 11) α-OM mAb 3E5; 12) α-OA pAb; 13) α-LY pAb.

a wide, strong band at M_r 32 000–46 000 and a weak band at M_r 68 000–70 000 developed. The former band has been identified by amino acid analysis as OM (1). Incubation with α -OM mAb 3E5 elicited bands identical to the reactions with α -OM pAb. A strong supplementary band at the M_r of LY (14 400) developed with α -LY pAb and a weaker one at the M_r of OA (37 000–45 000), confounding with OM developed by incubation with α -OA pAb. Incubations with α -OT pAb and α -KSTI pAb (negative control) revealed no visible bands.

Testing the three other protein preparations in the same manner gave similar results (data not shown). In particular, LY contained several contaminating proteins, including both OA and OT, as well as some protein bands smaller than LY. OT contained less contaminating proteins, among which OA was identified. OA appeared to be the most purified of all four proteins.

Purification of proteins

OT, OA, and OM were purified under identical conditions but with different NaCl gradients eluting the three proteins (Fig. 3). Beside the expected peaks from the particular proteins seen on the chromatograms, a few additional peaks were found in all four preparations. OM exists in at least two homologous variants, *var a* and *var b*, varying in *N*-glycosylation of the third domain (17). This protein was eluted in

two broad peaks over an interval of the gradient from 0.085 to 0.18 M NaCl. The two peaks were identified as OM by α -OM pAb and amino acid analyses. The two peaks were distinguished by the *N*-glucosylamine content determined by amino acid analyses, which identified the first eluting peak as OM *var a* and the later eluting peak as OM *var b* (data not shown). Iron-depleted OT was eluted at 0.05 M NaCl, a residue of Fe^{3+} -associated OT eluting as a peak at about 0.06 M NaCl. OA was eluted between 0.16 and 0.22 M. LY purified on a Mono S column was eluted at 0.16 M NaCl, in which impurities of the other more acidic proteins eluted even before the gradient started. For all four proteins, the gel filtration contributed to the purification of the proteins, in addition to serving as desalting steps.

Antibodies against purified proteins

Testing the pAbs raised on purified proteins in CIE of egg-white extract revealed immunoprecipitates containing specific antibodies against OM, OT, OA, and LY, respectively (Fig. 4). No supplementary immunoprecipitates were seen for α -OM, α -OA, and α -LY, indicating that highly specific antisera of these three proteins had been produced. One minor precipitate, apart from the characteristic OT precipitation peak, appeared on the CIE of α -OT pAb, indicating that this antiserum was not monospecific and would need purification if used for detailed studies.

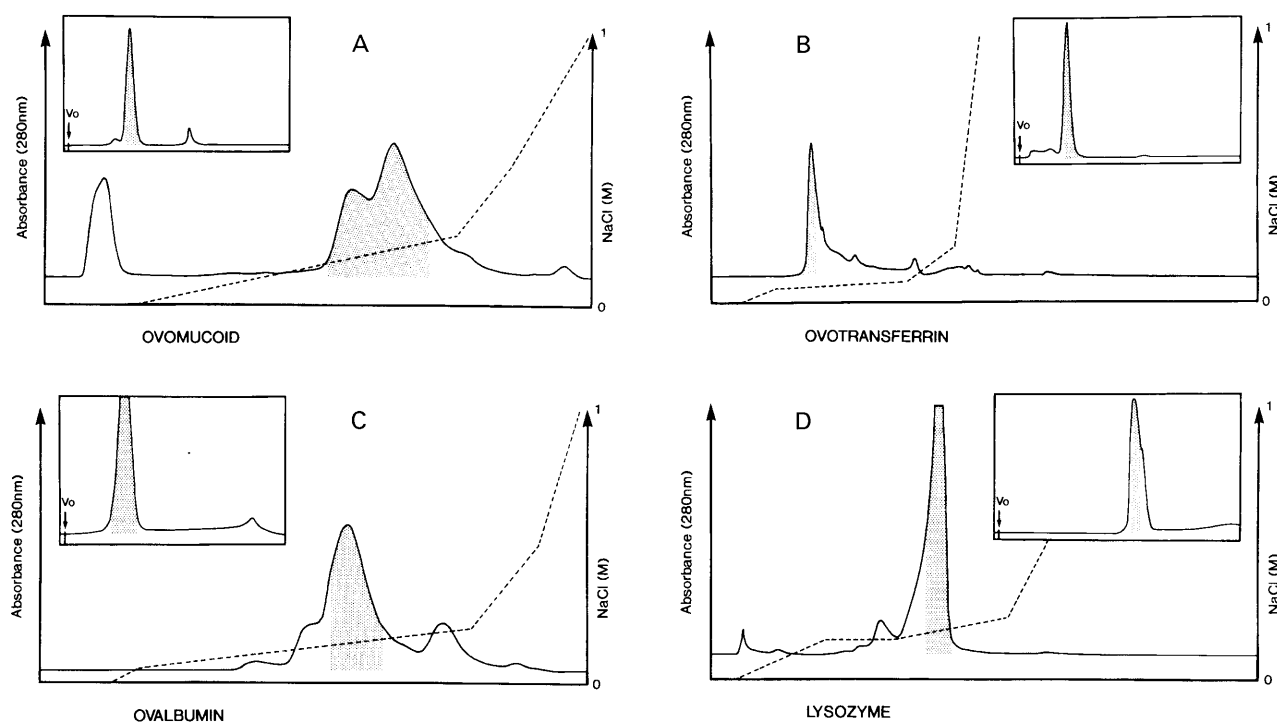


Fig. 3. Chromatograms from purification of A) ovomucoid on Q-Sepharose, B) ovotransferrin on Mono Q, C) ovalbumin on Q-Sepharose, and D) lysozyme on Mono S. Inserted chromatograms originate from respective gel filtrations on Sephacryl S-200 HR.

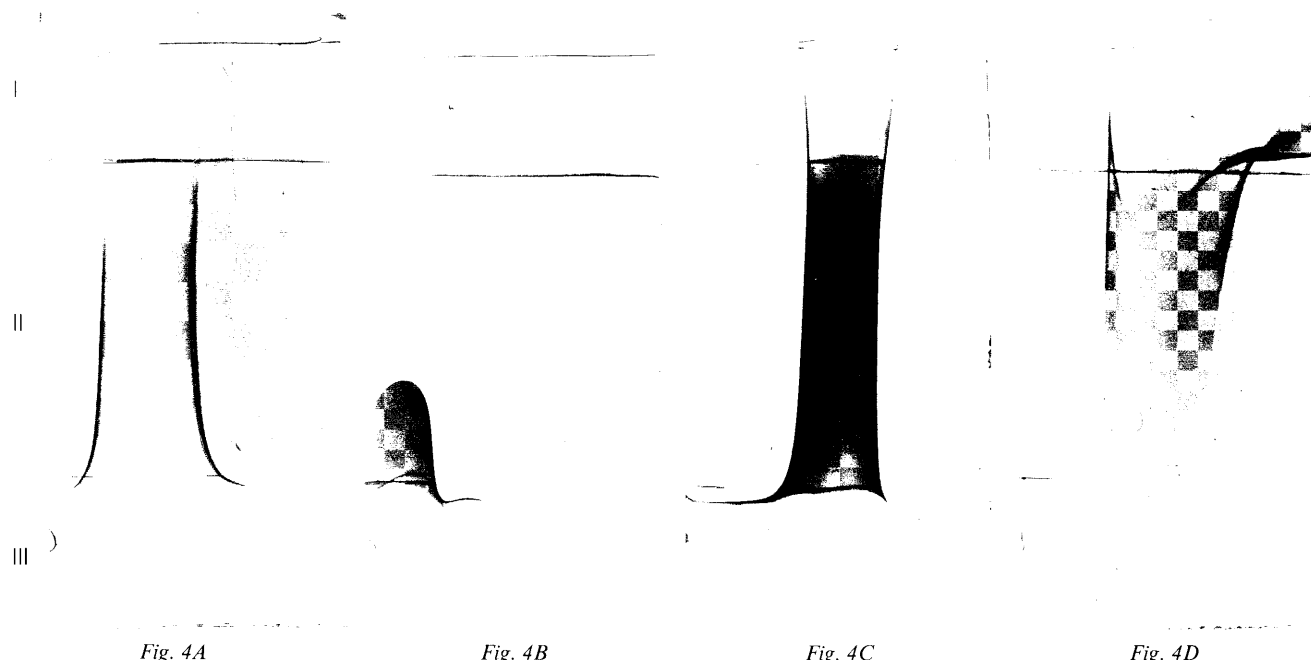


Fig. 4. Crossed immunoelectrophoreses of egg-white extract against pAbs raised on purified proteins. I and III) first dimension gel cut in application well; II) second dimension antibody-containing gel with A) α -OM pAb, B) α -OT pAb, C) α -OA pAb, or D) α -LY pAb.

Purity of purified proteins

CIE of the protein preparations with monospecific antibodies made it possible to test the purity of the proteins (Fig. 5). The differences between immunoprecipitation patterns on CIE of the crude and purified preparations of the individual proteins showed that the applied methods of purification had resulted in highly purified proteins. For OM, only one precipitate identified as OM was seen. The purified OA preparation showed one precipitate which was partially divided into two parts that coprecipitated along the edges and at the base lines. CIE of purified OT also yielded a precipitate internally divided into two areas, and a more blurred precipitate at the level of the well. Though several impurities in the LY preparations had been removed, CIE of purified LY revealed one major precipitate and one smaller distinct precipitate situated symmetrically under the main precipitate.

SDS-PAGE of the crude and purified proteins was done to identify any possible contaminants in the purified proteins (Fig. 6). OM was seen as a wide diffuse band from M_r 32 000–46 000, OT as a sharp narrow band at M_r 70 000, OA as a sharp but wider band at M_r 43 000, and LY as a sharp band at M_r 14 400; traces of the dimer were seen at M_r 28 000 (only visible on the original gels). In addition to the expected bands from the respective proteins, contaminating proteins were seen as weak bands in the crude preparation when the gel was stained with Coomassie brilliant blue. These bands were en-

hanced by silver staining of the gel, in which a few extra bands also appeared. In the purified preparations, very few traces of the contaminating proteins remained and visualization could be achieved only by the very sensitive silver-staining method.

Discussion

For detailed studies of the immune response to egg-white proteins, highly purified preparations are needed. Contamination of the LY preparation was evident in the initial studies with human sera on immunoblots (Fig. 1). However, human serum depending on specific antibody titers to the proteins would often fail to detect impurities. Moreover, contaminating proteins in the crude OM preparation have been detected by the reactions of proteins from the blot with antibodies specific for proteins other than OM (Fig. 2). Thus, specific antibodies against the individual proteins, as well as the entire egg-white extract, are a useful tool for determination of protein identity and purity.

OM variants consist primarily of *var a* glycosylated at five amino acids and *var b* missing one glycosylation at the third domain of the protein. *Var a* and *var b* eluted as the two major peaks in ion-exchange chromatography, as verified by amino acid analysis of *N*-glucosylamine content. In addition, variation in sialic acid content has been reported (34); this probably explains the tailing of the second peak in the chromatography, as evidenced by unchanged amino acid composition throughout the tail,

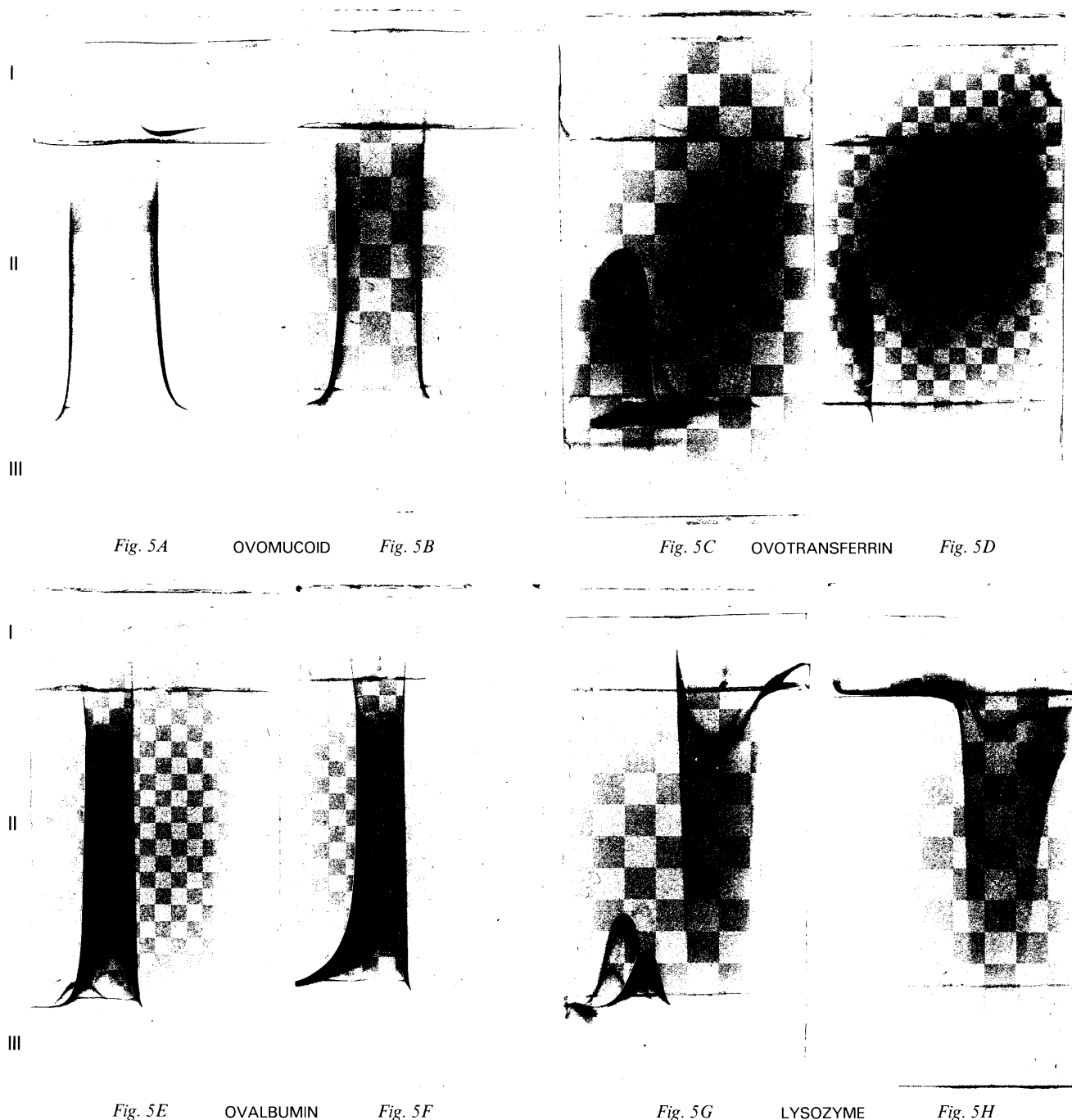


Fig. 5. Crossed immunoelectrophoreses of egg-white proteins before and after purification against α -EW pAb. Design as for Fig. 2. First dimension gels contained A) OM Sigma T-2011; B) purified OM; C) OT Sigma C-0880; D) purified OT; E) OA Sigma A-7641; F) purified OA; G) LY Sigma L-6876; H) purified LY.

unlike OM *var a/var b*. CIE of the purified OM indicated a high degree of purity, as evidenced by only one precipitate. It is possible that small levels of contaminants are not detected by CIE; accordingly, electrophoresis was done in SDS/polyacrylamide, which was subsequently stained with Coomassie brilliant blue and silver, respectively. Extensively glycosylated proteins such as OM are known to stain poorly with Coomassie (14), indicating that the rela-

tive impurity of the protein might have been even smaller than shown on the CIE and the Coomassie-stained gel. The silver-staining method is more sensitive; in particular, OM is more efficiently stained by this method. But still no bands could be seen at M_r 14400, 30000, or 43000, in contrast to the crude OM preparation. This crude OM preparation has been shown to contain LY and OA (Figs. 5 and 6), as verified by α -LY pAb and α -OA pAb, respectively

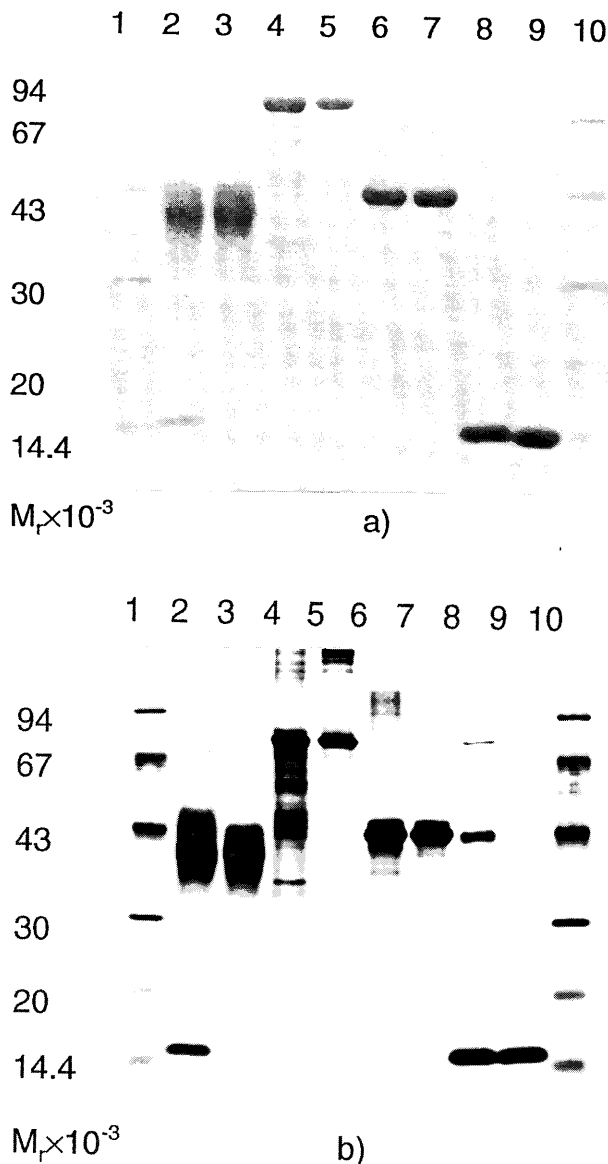


Fig. 6. SDS-PAGE of crude and purified proteins: A) stained with Coomassie brilliant blue, B) silver-stained. Lane 1) M_r marker; 2) OM Sigma T-2011; 3) purified OM; 4) OT Sigma C-0880; 5) purified OT; 6) OA Sigma A-7641; 7) purified OA; 8) LY Sigma L-6876; 9) purified LY.

(Fig. 2), and for LY also by amino acid analysis of this particular band (data not shown).

The CIE pattern of purified OA and OT indicated the presence of isoforms of the protein itself rather than remaining impurities. The partial identity of proteins possessing identical pI but slightly different precipitate patterns from those seen on CIE of OA and OT has been determined by rocket immunoelectrophoresis of mixtures of partially identical proteins (6). For OA, this identification is supported by the fact that the protein shows some heterogeneity in both serine phosphorylation (13) and glycosylation (11). In conclusion, the preparations of both OA and

OM attained a high degree of purity, while other proteins could no longer be detected by the applied methods.

As for OT, the purified preparation contained the major characteristic precipitation pattern from OT plus one minor precipitate below the OT peak. This could have been caused by trace amounts of the Fe-associated protein, which, although seen as a distinct peak in the ion-exchange chromatogram, never achieved base-line separation from the Fe-depleted OT peak. The Fe-depletion increased the pI of the protein from 5.78 to 6.73 (33), this probably would be reflected in a slowly running major precipitate and a faster running minor precipitate in the CIE pattern, as was, in fact, the case. Another possibility is that the precipitate derived from degraded OT. This is quite possible, because the crude OT preparation was observed to degrade spontaneously upon prolonged storage with repeated freezing and thawing (unpublished data). We conclude that in spite of possible OT-derived peptides remaining in the preparation, a high degree of purification was achieved.

As for LY, it seems impossible to decide by CIE whether the minor precipitate situated below the main precipitate originated from LY. Enlargement of the area near the well by prolongation of the electrophoresis in the first dimension was done (data not shown). This indicated similarity of the antigens in the two precipitates, because crossed precipitation lines did not occur between these two precipitates. In that case, the two precipitates could have been caused by dimerization of the protein, a phenomenon which is known to occur spontaneously (3). On the stained gels (Fig. 6) in lane 9 of LY, a trace of protein is seen at about M_r 28 000 (the size of the LY dimer), suggesting that the minor precipitate actually originated from LY. Identification of the minor precipitate could be achieved by amino acid analyses from blots of the solubilized antigen-antibody complexes on PVDF membrane.

Figs. 1 and 2 show the need of extensively purified proteins for studies of allergic reactions. The SDS-PAGE method partly solves the problem of contaminating proteins in the preparations, as exemplified by the fact that LY was well separated from the three other allergens tested. Yet protein bands can overlap because of slight or no difference in M_r , as exemplified by the bands of OM and OA so overlapping that distinction between these two proteins was impossible. The presence of contaminating proteins therefore adds great uncertainty to any variable measured even on SDS-PAGE blots. Evidently, such problems are increased whenever methods that do not separate the proteins are applied, e.g., dot blots, RAST, and ELISA. Furthermore, a large interbatch variation (quantitative as well as qualitative) between OM products of this

particular catalog number has been observed (unpublished data).

Our long-term project with OM, OT, OA, and LY is to describe the immune response not only on the protein level (cf. Ref. 1), but also on the epitope level for both T- and B-cell receptors. Since the development of the purification procedures described in the present paper, we have conducted several experiments in which the purified proteins were cleaved into mixtures of smaller peptides. It quickly became evident that even a 1% contamination with a protein resistant to digestion may cause severe bias in the interpretation of results from such digestions. In view of all these facts, it seems evident that great care should be taken to ensure the purity of egg-white proteins when they are used for studies of allergic reactions.

In conclusion, we have shown that it is possible in a few steps to purify all of the four proteins to an extent that impurities cannot be detected by the methods applied. From silver staining and CIE, we conclude that the proteins were so highly purified that, according to the highest minimum detection limits reported by the applied methods, the remaining impurities could be estimated to be less than 0.5–5 ng in the sample of 5 µg, i.e., less than 1/10 000–1/1000.

Moreover, the immunoblots of crude and purified OM against rabbit pAbs and α -OM mAbs and the IgE immunoblots of crude and purified LY probed with patient serum show that signals from all bands except OM and LY, respectively, had vanished. This fact indicates that problems with fragments originating from contaminating proteins are unlikely to occur even when fragmented or boiled proteins are tested. We therefore consider the purifications and characterizations satisfactory for future studies on the immunologic response of the fragmented proteins.

Acknowledgments

We wish to thank Ms Nina Milora and Ms Anne Blicher for skilful technical assistance.

References

1. AABIN B, POULSEN LK, EBBEHØJ K, et al. Identification of IgE-binding egg white proteins. Comparison of results obtained by different methods. *Int Arch Allergy Appl Immunol* 1994: (Submitted).
2. ANET J, BACK JF, BAKER RS, BARNETT D, BURLEY RW, HOWDEN MEH. Allergens in the white and yolk of hen's egg. *Int Arch Allergy Appl Immunol* 1985; 77: 364–71.
3. BACK JF. Changes in the proteins of the vitelline membrane in the hen's egg during storing. *Biochim Biophys Acta* 1984; 799: 319–21.
4. BLEUMINK E, YOUNG E. Studies of the atopic allergen in hen's egg. I. Identification of the skin reactive in egg-white. *Int Arch Allergy Appl Immunol* 1969; 35: 1–19.
5. BLUM H, BEIER H, GROSS HJ. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 1987; 8: 93–9.
6. BOCK E, AXELSEN NH. Partial antigenic identity. *Scand J Immunol* 1983; 17 (Suppl. 10): 183–7.
7. BOCK SA. A critical evaluation of clinical trials in adverse reactions to foods in children. *J Allergy Clin Immunol* 1986; 78: 165–73.
8. BOCK SA, SAMPSON HA, ATKINS FM, et al. Double-blind, placebo-controlled food challenge (DBPCFC) as an office procedure. A manual. *J Allergy Clin Immunol* 1988; 82: 986–97.
9. BONDE M, PONTOPPIDAN H, PEPPER DS. Rigid hydrazone/hydrazide modified solid supports for site directed immobilization of antibodies. *Prep Chromatogr* 1991; 1: 269–77.
10. CLARKE HGM, FREEMAN T. Quantitative immunoelectrophoresis of human serum proteins. *Clin Sci* 1968; 35: 403–13.
11. CUNNINGHAM LW, CLOUSE RW, FORD JD. Heterogeneity of the carbohydrate moiety of crystalline ovalbumin. *Biochim Biophys Acta* 1963; 78: 379–81.
12. EBBEHØJ K. Characterization of allergens from hen's egg white. PhD Thesis, Department of Biochemistry and Nutrition, Technical University of Denmark, 1994.
13. EGELANDSDAL B. Heat-induced gelling in solutions of ovalbumin. *J Food Sci* 1980; 45: 570–3.
14. FOUNTOULAKIS M, JURANVILLE J-F, MANNEBERG M. Comparison of the Coomassie brilliant blue, bicinchoninic acid and Lowry quantitation assays, using non-glycosylated and glycosylated proteins. *J Biochem Biophys Methods* 1992; 24: 265–74.
15. HOFFMAN DR. Immunochemical identification of the allergens in egg white. *J Allergy Clin Immunol* 1983; 71: 481–6.
16. JAROLIM E, MATTHIESEN F, STAHL SKOV P, TEJKL M, ØSTERBALLE O, POULSEN LK. Hyposensitization therapy with whole pollen extract or purified allergens monitored by immunoblotting. *Int Arch Allergy Appl Immunol* 1990; 92: 254–64.
17. KATO I, SCHRODE J, KOHR WJ, LASKOWSKI MJR. Chicken ovomucoid: determination of its amino acid sequence, determination of the trypsin reactive site, and preparation of all three of its domains. *Biochemistry* 1987; 26: 193–201.
18. KYHSE-ANDERSEN J. Electroblotting of multiple gels: a simple horizontal apparatus without buffer tank for electrophoretic transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Methods* 1984; 10: 203–9.
19. LANGELAND T. A clinical and immunological study of allergy to hen's egg white. II. Antigens in hen's egg white studied by crossed immunoelectrophoresis (CIE). *Allergy* 1982; 37: 323–33.
20. LANGELAND T. A clinical and immunological study of allergy to hen's egg white. III. Allergens in hen's egg white studied by crossed radio-immunoelectrophoresis (CRIE). *Allergy* 1982; 37: 521–30.
21. LANGELAND T. A clinical and immunological study of allergy to hen's egg white. I. A clinical study of egg allergy. *Clin Allergy* 1983; 13: 371–82.
22. LANGELAND T, HARBITZ O. 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. *Allergy* 1983; 38: 131–9.
23. MATSUDAIRA P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* 1987; 262: 10035–8.
24. MOEREMANS M, DANELLIS G, DE MEY J. Sensitive colloidal metal (gold or silver) staining of protein blots on nitrocellulose membranes. *Anal Biochem* 1985; 145: 315–21.
25. MYGIND N. Foods that cause allergy: milk and egg most important. In: MYGIND N, ed. *Essential allergy: an illustrated text for students and specialists*. Oxford: Blackwell Scientific, 1986: 144–9.
26. NØRGAARD A, BINDSLEV-JENSEN C. Egg and milk allergy in adults. *Allergy* 1992; 47: 503–9.

27. RAMLAU J. Use of secondary antibodies for visualization of bound primary reagents in blotting procedures. *Electrophoresis* 1987; 8: 398–402.
28. SAMPSON HA. IgE-mediated food intolerance. *J Allergy Clin Immunol* 1988; 81: 495–504.
29. SAMPSON HA, COOKE SK. Food allergy and the potential allergenicity-antigenicity of microparticulated egg and cow's milk proteins. *J Am Coll Nutr* 1990; 9: 410–17.
30. SAMPSON HA, McCASKILL CC. Food hypersensitivity and atopic dermatitis: evaluation of 113 patients. *J Pediatr* 1985; 107: 669–75.
31. SCHÄGGER H, VON JAGOW G. Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal Biochem* 1987; 166: 368–79.
32. WEEKE B. Crossed immunoelectrophoresis. *Scand J Immunol* 1973; 2 (Suppl. 1): 47–56.
33. WENN RV, WILLIAMS J. The isoelectric fractionation of hen's-egg ovotransferrin. *Biochem J* 1968; 108: 69–74.
34. YAMASHITA K, TACHIBANA Y, HITOL A, KOBATA A. Sialic acid-containing sugar chains of hen ovalbumin and ovomucoid. *Carbohydr Res* 1984; 130: 272–88.