

Affinity Purification of Egg-White Allergens for Improved Component-Resolved Diagnostics

Henrik Everberg^a Peter Brostedt^a Hans Öman^b Svante Bohman^a
Robert Movérare^{a, c}

^aPhadia AB, ^bMIAB and ^cDepartment of Medical Sciences, Respiratory Medicine and Allergology, Uppsala University, Uppsala, Sweden

Key Words

Egg allergy · Ovomucoid · Ovalbumin · Conalbumin · Lysozyme · Affinity purification · ImmunoCAP · Chimeric IgE antibody

Abstract

Background: Egg is a common cause of food-allergic reactions, especially among young children. Some egg-allergic patients do, however, tolerate heated egg products and component-resolved diagnostics (CRD) may facilitate prediction of different disease manifestations. Commercially available preparations of the egg-white allergens, ovomucoid, ovalbumin, conalbumin and lysozyme, have been reported to contain impurities which interfere with accurate CRD. **Methods:** Commercial preparations of the 4 egg-white allergens were characterized using allergen-specific monoclonal chimeric human/mouse IgE antibodies in experimental ImmunoCAP® tests. Further purification of commercial ovomucoid, ovalbumin and conalbumin preparations was performed by chromatography based on affinity to monoclonal antibodies. Purity was monitored by size exclusion chromatography, SDS-PAGE, Western blotting and experimental ImmunoCAP tests using allergen-specific chimeric IgE antibodies. IgE reactivity to the highly purified egg com-

ponents was analyzed in 83 samples from egg white-sensitized individuals. **Results:** Preparations of commercially available ovomucoid, ovalbumin and conalbumin were found to contain other egg allergens which were removed by chromatographic purification. No impurities were detected in the commercial lysozyme preparation. Previously unknown complexes between the target allergens and contaminating allergens were detected and removed by affinity chromatography. IgE reactivity to ovalbumin was most common in the analyzed samples (87%), followed by ovomucoid (72%), conalbumin (69%) and lysozyme (58%). **Conclusions:** In this study we demonstrate the advantage of using monoclonal antibodies for purification, and monoclonal chimeric IgE antibodies for characterization, of egg allergens intended for CRD. Our study also established that ovalbumin, ovomucoid, conalbumin and lysozyme are all major allergens.

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Introduction

Allergic reactions caused by ingestion of egg are estimated to occur in up to 1.7% of infants and young children [1–3]. Most of the reactions are caused by allergens present in the egg white [4]. Approximately 80% of the

total protein content in the egg white is made up of the 4 allergens ovomucoid (Gal d 1, approx. 11%), ovalbumin (Gal d 2, approx. 54%), conalbumin, also known as ovotransferrin (Gal d 3, approx. 12%), and lysozyme (Gal d 4, approx. 3%) [5]. Ovomucoid is a highly glycosylated, heat-resistant serine protease inhibitor [5, 6], and a potent egg allergen [7, 8]. Ovalbumin, the major protein constituent of egg white, is a phosphorylated glycoprotein with unknown biological function [9]. In addition, ovalbumin is heat labile and undergoes conformational changes to form the more stable, and possibly less allergenic, S-ovalbumin upon exposure to elevated temperature [9–10]. Conalbumin is an iron-binding glycoprotein and lysozyme is a glycosidase commonly used as a food preservative due to its antibacterial properties [5].

Component-resolved diagnostics (CRD) is helpful to predict different disease manifestations. For example, ovomucoid-specific IgE antibodies have been suggested as markers of allergy to heated egg [11]. While a prerequisite for accurate CRD is the use of highly purified allergen preparations for testing, commercially available preparations of ovomucoid, ovalbumin, conalbumin and lysozyme, have been reported to contain other egg-white allergens [12–14]. Further purification of such crude egg allergen preparations has been described in a number of publications using different chromatography techniques [12–16]. Characterization of crude and purified allergen preparations have generally been performed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), IgE immunoblotting and IgE ELISA using human serum samples. However, SDS-PAGE and immunoblotting have limited sensitivity for detection of low amounts of contaminants, and human serum samples are of limited utility for allergen-specific detection due to their polyclonal nature. Thus, there is a need for improved characterization methods in terms of sensitivity and specificity for the major egg-white allergens to ensure accurate CRD of egg-allergic individuals.

The aim of this study was to use the superior sensitivity and specificity provided by monoclonal chimeric human/mouse IgE antibodies for characterization of the 4 major egg-white allergens coupled to experimental ImmunoCAP® tests. Furthermore, efficient protocols for affinity purification of crude ovomucoid, ovalbumin and conalbumin were developed based on monoclonal antibodies (mAbs). The highly purified allergens, suitable for CRD, were used in the analysis of IgE reactivity in samples from egg white-sensitized individuals using ImmunoCAP.

Material and Methods

Crude Egg Allergens and Other Reagents

Preparations of crude ovomucoid (T2011), ovalbumin (A5503), conalbumin (C0880) and lysozyme (L6876) were purchased from Sigma-Aldrich (St. Louis, Mo., USA). Extinction coefficients were derived from ExPASy (ovomucoid 0.47, accession No. P01005; ovalbumin 0.74, P01012; conalbumin 1.1, P02789; lysozyme 2.64, P00698). The egg-white allergen-specific mAbs used (anti-ovomucoid mAb 5B6, anti-ovalbumin mAb 5E2, anti-conalbumin mAb 5F11 and anti-lysozyme mAb 2G5) were developed in-house [17]. Anti-mouse IgG antibody conjugated with horseradish peroxidase was purchased from GE Healthcare (Uppsala, Sweden). Chimeric human/mouse IgE antibodies against ovomucoid, ovalbumin, conalbumin and lysozyme used in the study were based on in-house egg-white allergen-specific mAbs and developed as described elsewhere [18]. The chimeric IgE antibodies consist of light chains (V_L and C_L domains) and V_H domains of mouse origin, and heavy-chain constant domains of human IgE origin ($C\epsilon_1$ – $C\epsilon_4$). Thus, they appear as human IgE antibodies in IgE-specific immunoassays and maintain the allergen specificity of the mAbs from which they originate [19].

Biochemical Characterization

Analytical size exclusion chromatography (SEC) was performed on a Superdex 75 10/300 GL column (GE Healthcare) in 20 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 0.02% NaN_3 . Molecular weight calibration of the column was performed using a high and low molecular weight calibration kit (GE Healthcare).

SDS-PAGE was performed on NuPAGE 4–12% Bis Tris Gel in MES running buffer (Invitrogen, Carlsbad, Calif., USA). Prior to loading, protein samples were prepared under nonreducing conditions with NuPAGE LDS Sample buffer (Invitrogen) and heated at 97°C for 5 min. For reducing conditions, a final concentration of 10 mM dithiothreitol (DTT) was added to the samples. The gels were loaded with 0.5 µg protein per lane. Molecular weight calibration was achieved using Mark 12 Unstained Standard (Invitrogen). The gels were silver stained according to Blum et al. [20].

Proteins were blotted from SDS-PAGE (see above) onto polyvinylidene difluoride membrane (Invitrogen). Blocking was performed with powdered milk, followed by incubation with the allergen-specific mAb. Detection of bound mAbs was performed using anti-mouse IgG conjugated with horseradish peroxidase and ECL Plus substrate (GE Healthcare). Scanning of the membrane was performed with a Typhoon TM 9410 (GE Healthcare).

In-gel digestion was performed according to Shevchenko et al. [21] and MALDI-TOF mass spectrometry analysis was performed using a Bruker Autoflex II (Bruker Daltonic, Bremen, Germany).

Purification of Egg-White Allergens

Crude ovomucoid was dissolved to a concentration of 20 mg/ml in 50 mM sodium phosphate buffer, pH 7.0, and cation-exchange chromatography was performed on a Capto S column (GE Healthcare). Residual lysozyme was removed by affinity adsorption chromatography using anti-lysozyme (mAb 2G5) coupled to NHS-activated Sepharose HP according to the supplier's instruction (GE Healthcare). Crude ovalbumin (20 mg/ml) and conalbumin (5 mg/ml) was dissolved in 20 mM sodium phosphate buffer, pH 8.0, and purified by affinity adsorption chromatography with

contaminant-specific mAbs. For purification of ovalbumin, anti-ovomucoid (mAb 5B6) and anti-conalbumin (mAb 5F11) were used. For purification of conalbumin, anti-ovomucoid (mAb 5B6) and anti-lysozyme (mAb 2G5) were used. The purified ovomucoid, ovalbumin and conalbumin were all collected in the flow-through fractions and the adsorbed contaminants were eluted from the affinity columns in 0.1 M Glycine-HCl buffer pH 3.0 and immediately neutralized with 1 M K₃PO₄ for further analysis. Protein concentrations were determined by absorbance at 280 nm and extinction coefficients from ExPASy. The proteins were frozen at -20°C until use.

Experimental Egg-White Component ImmunoCAP Tests

Crude ovomucoid, ovalbumin, conalbumin and lysozyme preparations, as well as the purified allergen preparations, were coupled to experimental ImmunoCAP tests. Chimeric human/mouse IgE antibodies against all 4 egg-white allergens were used in single measurements for evaluation of the specificity of the tests. Cutoff points were set to 0.35 kU_A/l. Negative samples spiked with up to 10,000 kU/l myeloma IgE were used to assess unspecific binding.

Specific IgE Measurements (CRD)

Eighty-three samples from egg white-sensitized individuals (specific IgE to egg white ImmunoCAP >0.35 kU_A/l) from Sweden (n = 25), Spain (n = 16), Italy (n = 3), Germany (n = 4), Japan (n = 14) and the United States (n = 21) were analyzed using experimental ImmunoCAP tests based on the highly purified egg-white allergens. For comparison, the samples were also analyzed with ImmunoCAP tests with crude preparations of ovomucoid, ovalbumin and conalbumin. All specific IgE antibody analyses were performed using ImmunoCAP (Phadia AB, Uppsala, Sweden). All samples were collected under the approval of the local ethics committee at each center.

Statistical Analysis

Wilcoxon signed-rank test and Fisher's exact test (both two-tailed) were used for pairwise comparisons of continuous parameters (specific IgE concentrations) and categorical data (prevalence for IgE reactivity) between experimental ImmunoCAP tests with allergen components. Spearman's rank order correlation coefficient (r_s) was used to measure the strength of the relationship between specific IgE antibody levels obtained by different tests. Only p values less than 0.05 were considered significant.

Results

Experimental ImmunoCAP Tests with Crude Egg-White Allergen Preparations

Experimental ImmunoCAP tests were prepared with each allergen in order to characterize and estimate the purity of preparations of crude ovomucoid, ovalbumin, conalbumin and lysozyme. The specificity of each test was evaluated using monoclonal human/mouse chimeric IgE antibodies against all 4 allergens (table 1). The experimental test based on crude ovomucoid demonstrated

Table 1. ImmunoCAP analysis of commercially available (crude) and purified preparations of egg-white allergens using monoclonal chimeric human/mouse IgE antibodies

Allergen preparation	Chimeric IgE antibody			
	a-ovo	a-ova	a-con	a-lys
Crude ovomucoid	57.8	0.04	0.04	53.2
IEX-purified ovomucoid	55.7	0.02	0.09	1.4
IEX + affinity-purified ovomucoid	56.4	0.04	0.12	0.08
Crude ovalbumin	37.4	93.6	0.35	0.03
Affinity-purified ovalbumin	0.10	81.7	0.05	0.03
Crude conalbumin	0.29	0.02	80.5	2.0
Affinity-purified conalbumin	0.08	0.01	85.0	0.02
Commercial lysozyme	0.12	0.04	0.07	93.0

IEX = Ion exchange chromatography; a-ovo = anti-ovomucoid; a-ova = anti-ovalbumin; a-con = anti-conalbumin; a-lys = anti-lysozyme. Levels of IgE binding are expressed as kU_A/l.

a significant binding of the lysozyme-specific chimeric IgE antibody, indicating that the crude ovomucoid preparation contained a substantial amount of lysozyme. Similarly, the crude ovalbumin was found to contain ovomucoid and, to a lesser extent, conalbumin. The crude conalbumin was found to contain both ovomucoid and lysozyme. The crude lysozyme preparation was considered to have a satisfactory purity as only binding of lysozyme-specific chimeric IgE antibody was detected.

Characterization and Purification of Ovomucoid

The crude ovomucoid was analyzed by SEC (fig. 1, hatched line), where the dominating peak containing ovomucoid was observed at an elution volume corresponding to a molecular weight of approximately 55 kDa although the theoretical molecular weight of ovomucoid is 20 kDa. The lysozyme-containing peak was observed at an elution volume corresponding to a lower apparent molecular weight (approx. 3 kDa) than expected for lysozyme (14 kDa). SDS-PAGE displayed a diffuse band at approximately 40 kDa and a band at approximately 14 kDa (fig. 2a, lane 1). Western blotting using the lysozyme-specific mAb 2G5 verified the identity of lysozyme at 14 kDa (fig. 2b, lane 1).

The bulk of contaminating lysozyme was removed from the crude ovomucoid preparation by cation-exchange chromatography at pH 7 where ovomucoid was collected in the flow-through material while lysozyme was retained on the column as shown by SEC analysis

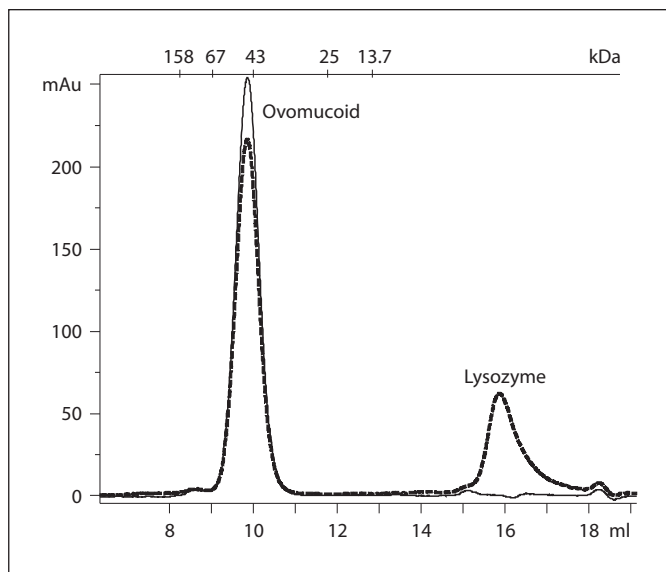


Fig. 1. SEC of crude ovomucoid before (hatched line) and after (solid line) ion-exchange chromatography.

(fig. 1, solid line). The eluted lysozyme was observed at 14 kDa on SDS-PAGE and Western blotting using the anti-lysozyme mAb (fig. 2a, b, lane 3). However, after coupling the ovomucoid preparation purified by ion exchange to ImmunoCAP, a response corresponding to 1.4 kU_A/l could still be detected with the lysozyme-specific chimeric IgE antibody used at 93 kU_A/l (table 1). Western blotting of the ion exchange-purified ovomucoid preparation using the lysozyme-specific mAb detected a diffuse band with an apparent molecular weight of approximately 50–55 kDa on the gel (fig. 2b, lane 2).

After further purification of the ion exchange-purified ovomucoid preparation by anti-lysozyme affinity adsorption chromatography, the band at 50–55 kDa was no longer visible in Western blotting with the anti-lysozyme mAb (fig. 2b, lane 4) and no further binding of the lysozyme-specific chimeric IgE antibody could be detected in ImmunoCAP (table 1). SDS-PAGE of the material eluted from the anti-lysozyme affinity column revealed bands at both 14 and 50–55 kDa (fig. 2a, lane 5). The same pattern was observed under reducing conditions (results not shown). Western blotting using the anti-lysozyme mAb resulted in bands at the same positions (fig. 2b, lane 5). Also, the ovomucoid-specific mAb reacted against the band at 50–55 kDa, indicating the presence of a lysozyme-ovomucoid complex which was removed by affinity adsorption chromatography (fig. 2c, lane 5). The pres-

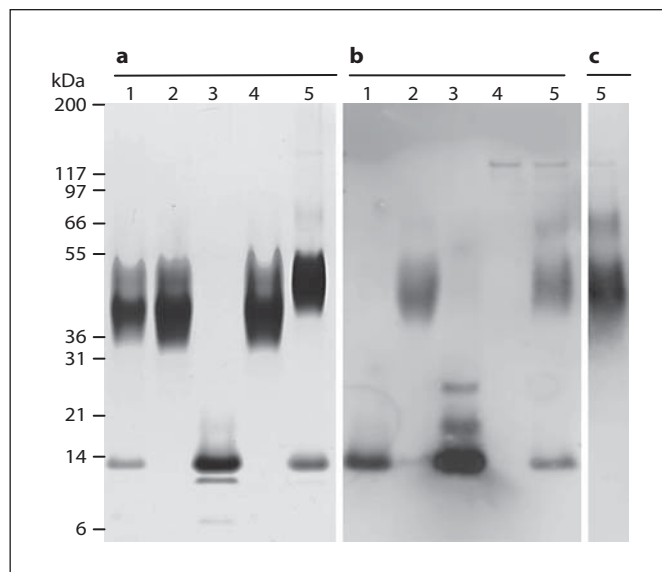


Fig. 2. SDS-PAGE of fractions from ovomucoid purification (**a**) and Western blotting with lysozyme-specific (**b**) and ovomucoid-specific (**c**) mAbs. Lane 1: crude ovomucoid preparation; lane 2: flow-through fraction from ion-exchange chromatography; lane 3: fraction eluted from ion-exchange chromatography column; lane 4: flow-through fraction after ion-exchange and anti-lysozyme affinity adsorption chromatography; lane 5: fraction eluted from anti-lysozyme column.

ence of lysozyme in the band at 50–55 kDa, corresponding to the lysozyme-ovomucoid complex, was confirmed with mass spectrometry analysis after in-gel digest. The purification protocol presented here resulted in a highly purified preparation of ovomucoid with a final protein yield of 90% of the starting material.

Characterization and Purification of Ovalbumin

SEC analysis of the crude ovalbumin preparation resulted in one dominating peak with a molecular weight of approximately 55 kDa (the theoretical molecular weight of ovalbumin is 43 kDa) and smaller peaks of higher molecular weight (fig. 3, hatched line). The dominating forms of ovomucoid and ovalbumin migrated to overlapping positions on SDS-PAGE (fig. 4a, lane 1). Western blotting analysis with the anti-ovomucoid (5B6) and anti-conalbumin (5F11) mAbs verified the presence of the contaminants in the crude preparation of ovalbumin (fig. 4b, c, lane 1).

Affinity adsorption chromatography with anti-ovomucoid mAb 5B6 and anti-conalbumin mAb 5F11 resulted in efficient removal of the contaminating proteins

from the crude ovalbumin preparation. The purified ovalbumin (flow-through fraction) and the removed contaminants were analyzed by SEC and the dominating peaks eluted at volumes corresponding to approximately 55 (ovalbumin) and 48 kDa (ovomucoid), respectively, with possible dimeric forms at approximately 106 and 100 kDa (fig. 3, solid line and dotted line, respectively). Ovalbumin was found to be present as 4 bands on nonreducing SDS-PAGE (fig. 4a, lane 2) as well as under reducing conditions (results not shown). A major band at approximately 43 kDa, bands at approximately 95 kDa, 130 kDa and a faint band at approximately 160 kDa were observed. Western blotting with the anti-ovalbumin mAb 5E2 confirmed the identity of these bands as monomeric and aggregated forms of ovalbumin (fig. 4d, lane 2).

The removed contaminants were observed as a diffuse major band with its center at approximately 35 kDa, and bands at 63, 110 and 150 kDa (fig. 4a, lane 3). Western blotting with the ovomucoid-specific mAb revealed bands mainly at 63 and 110 kDa, while the monomeric form was detected at approximately 40 kDa as a faint diffuse band (fig. 4b, lane 3). The contaminating conalbumin in the crude ovalbumin preparation was not seen in SEC or SDS-PAGE, but was readily detected in Western blotting with the conalbumin-specific mAb (fig. 4c). Conalbumin appeared in the high molecular weight region of the blot, with dominating bands at approximately 110 and 150 kDa and a number of faint bands. A faint band was also detected at 63 kDa. The ovalbumin-specific mAb bound to several bands in the material eluted from the affinity columns indicating the presence of ovalbumin in complexes with ovomucoid and/or conalbumin (fig. 4d, lane 3). The described affinity purification resulted in a highly purified preparation of ovalbumin with a protein yield of approximately 66% of the starting material.

Characterization and Purification of Conalbumin

SDS-PAGE analysis of the crude conalbumin (theoretical molecular weight: 76 kDa) displayed a large number of bands with high apparent molecular weights (fig. 5a, lane 1). Western blotting of the crude preparation with the ovomucoid- and lysozyme-specific mAbs displayed very faint bands at the same positions as the proteins eluted from the anti-ovomucoid and anti-lysozyme affinity columns (fig. 5b, c, lanes 1 and 3).

Purification of conalbumin was performed by affinity adsorption chromatography with anti-lysozyme mAb 2G5 and anti-ovomucoid mAb 5B6. SDS-PAGE of the purified conalbumin (fig. 5a, lane 2) displayed a large num-

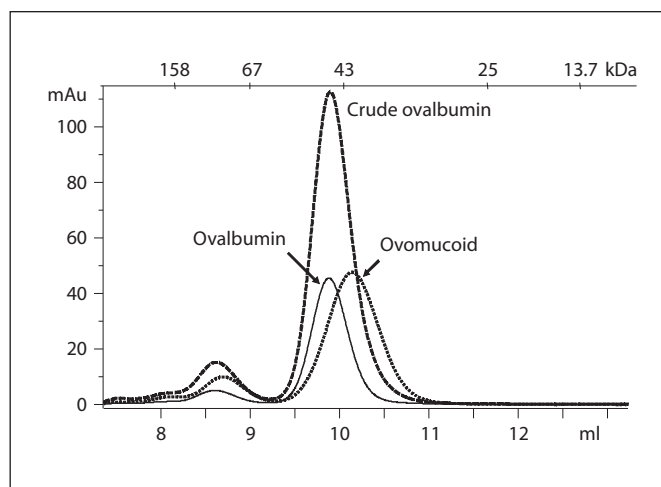


Fig. 3. SEC of crude ovalbumin (hatched line), purified ovalbumin (solid line) and removed ovomucoid (dotted line) after anti-ovomucoid and anti-conalbumin affinity adsorption chromatography.

ber of bands with a pattern very similar to the crude conalbumin, and the same pattern was observed also under reducing conditions (results not shown). Western blotting analysis of the material eluted from the anti-lysozyme and anti-ovomucoid affinity columns revealed lysozyme-containing bands at approximately 50, 75 and 100 kDa together with a number of bands with a high apparent molecular weight (fig. 5b, lane 3). Ovomuroid was detected at approximately 95, 160 and 210 kDa (fig. 5c, lane 3). The final protein yield of purified conalbumin was 95% of the starting material.

Experimental ImmunoCAP Tests with Purified Egg-White Allergens

Experimental ImmunoCAP tests based on the highly purified egg-white allergen components (including the commercially available lysozyme) were developed and their specificity was confirmed using chimeric IgE antibodies against the 4 major egg-white allergens (table 1). A slight decrease in binding of the ovalbumin-specific chimeric IgE antibody was observed after purification. No unspecific binding was observed with negative samples (data not shown).

Prevalence of IgE Reactivity to Egg-White Components

Eighty-three samples from egg white-sensitized subjects were analyzed for IgE binding to the highly purified egg-white allergens (table 2). The difference in prevalence

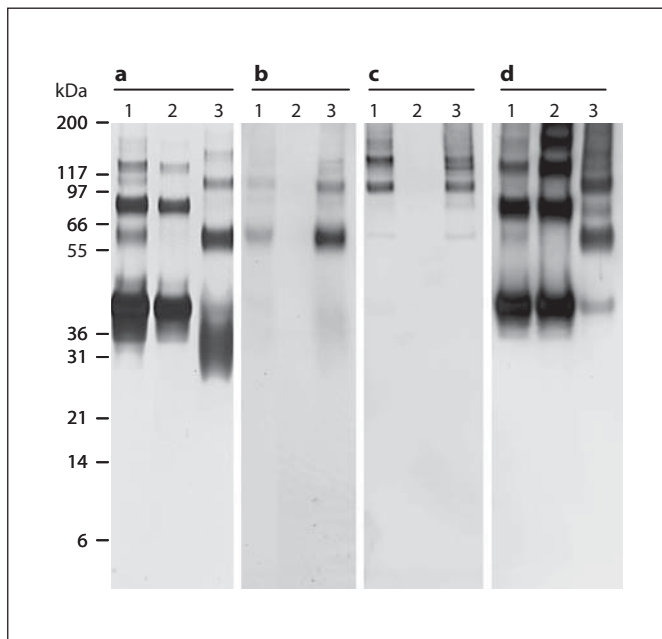


Fig. 4. SDS-PAGE of fractions from ovalbumin purification (**a**) and Western blotting with ovomucoid-specific (**b**), conalbumin-specific (**c**) and ovalbumin-specific (**d**) mAbs. Lane 1: crude ovalbumin; lane 2: flow-through fraction after anti-ovomucoid and anti-conalbumin affinity adsorption chromatography; lane 3: proteins eluted from the affinity columns.

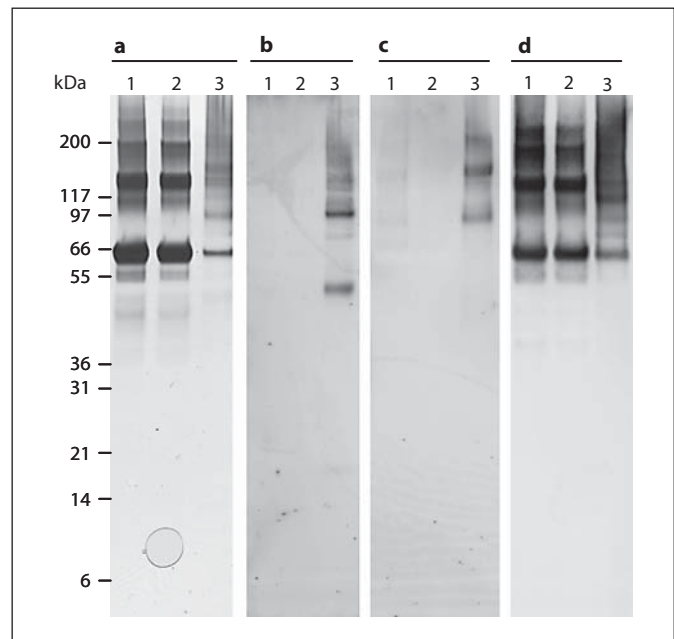


Fig. 5. SDS-PAGE of fractions from conalbumin purification (**a**) and Western blotting with lysozyme-specific (**b**), ovomucoid-specific (**c**) and conalbumin-specific (**d**) mAbs. Lane 1: crude conalbumin; lane 2: flow-through fraction after anti-ovomucoid and anti-lysozyme affinity adsorption chromatography; lane 3: proteins eluted from the affinity columns.

was significant between ovalbumin (87%) and conalbumin (69%, $p < 0.05$), and between ovalbumin and lysozyme (58%, $p < 0.0001$). The number of positive test results per patient varied from 0 ($n = 3$) to 4 ($n = 34$) of the egg-white allergen components studied (mean: 2.86 positive tests).

The specific IgE levels observed to highly purified ovalbumin were higher than to ovomucoid, conalbumin and lysozyme (table 2, all comparisons: $p < 0.0001$). The specific IgE levels to highly purified ovomucoid and conalbumin were higher than to lysozyme (both: $p < 0.0001$).

When coupled to ImmunoCAP, crude ovomucoid, ovalbumin and conalbumin showed higher IgE binding with samples from egg white-sensitized subjects compared to the highly purified preparations, resulting in a larger number of positive test results and higher apparent IgE antibody concentrations (table 2).

The specific IgE levels to the 4 allergen components weakly correlated with each other ($r_s = 0.39$ – 0.78 , $p < 0.0001$ – 0.0003). The highest correlation was found between the specific IgE levels to ovalbumin and conalbumin.

Discussion

In this study, sensitive and specific methods were developed for characterization of the egg-white allergens ovomucoid, ovalbumin, conalbumin and lysozyme intended for use in CRD. Since commercial ovomucoid, ovalbumin and conalbumin were found to be contaminated with other egg-white allergens, purification protocols were developed based on affinity to mAbs. Using the highly purified allergens, experimental ImmunoCAP tests were developed and used for analyses of specific IgE antibody responses in a set of samples from egg-sensitized individuals.

The crude ovomucoid preparation was found to be heavily contaminated with lysozyme as confirmed by SEC, SDS-PAGE and Western blotting with allergen-specific mAbs. The observed elution volume of ovomucoid (corresponding to a molecular weight of approx. 55 kDa) differed from the theoretical molecular weight based on the amino acid sequence (20 kDa) in the SEC analysis and this is most likely explained by the high carbohydrate content of this glycoprotein [5, 6]. The lysozyme-contain-

Table 2. Comparison of IgE binding to commercially available (crude) and purified preparations of egg-white allergen components in 83 egg white-sensitized individuals using ImmunoCAP

	Ovomucoid	Ovalbumin	Conalbumin	Lysozyme
Crude preparation				
Number and percentage ^a	75 (90%)	75 (90%)	73 (88%)	48 (58%)
Concentration with range, kU _A /l	2.64 (<0.35–>100)	4.83 (<0.35–>100)	3.59 (<0.35–>100)	0.52 (<0.35–61.3)
Purified preparation				
Number and percentage ^a	60 (72%)*	72 (87%)	57 (69%)*	NA ^b
Concentration with range, kU _A /l	1.60 (<0.35–62.6)**	3.46 (<0.35–>100)**	1.75 (<0.35–>100)**	

^a Cut-off: 0.35 kU_A/l; ^b Purification not needed. * $p < 0.01$ comparison between commercial and purified preparation (Fisher's exact test). ** $p < 0.001$ comparison between commercial and purified preparation (Wilcoxon signed rank test).

ing peak was observed at an elution volume corresponding to a low apparent molecular weight, inconsistent with the theoretical molecular weight of lysozyme. Such anomalous behavior has previously been explained as a result of interactions between basic proteins (the theoretical isoelectric point of lysozyme is 9.3) and the chromatography media [22].

Commercially available preparations of ovomucoid, purified by ion-exchange chromatography and SEC, have previously been reported to have a purity of >99% as monitored by SDS-PAGE and IgE immunoblotting with serum samples [13–14]. The results presented here confirm that ion-exchange chromatography purification resulted in a pure preparation of ovomucoid as judged by SEC and SDS-PAGE. However, after coupling the ion exchange-purified ovomucoid preparation to ImmunoCAP, binding of the lysozyme-specific chimeric IgE antibody was still detected when a sample containing high levels of lysozyme-specific chimeric IgE antibody was analyzed. Western blotting with a lysozyme-specific mAb detected a diffuse band with an apparent molecular weight of approximately 50–55 kDa, suggesting that a fraction of the lysozyme was aggregated with ovomucoid to form a lysozyme-ovomucoid complex not removed by ion-exchange chromatography. The hypothesis of a lysozyme-ovomucoid complex was further strengthened by Western blotting using a ovomucoid-specific mAb which resulted in a band at the same position. Furthermore, the presence of lysozyme in the band corresponding to the lysozyme-ovomucoid complex was also verified by mass spectrometry analysis.

The formation of a lysozyme-ovomucoid complex, as well as the other protein complexes found in this study, may be a result of the treatment during large-scale purification of the commercially available egg-white allergen components. The observation that the lysozyme-ovomu-

coid complex was found to be intact during denaturing and reducing conditions suggests that the components of the complex are strongly bound to each other. Even though the lysozyme-ovomucoid complex was present in a very small amount in the ovomucoid preparation, it was enough to detect IgE antibodies to lysozyme in ImmunoCAP, demonstrating that even a minute contamination of another allergen component, may lead to misinterpreted conclusions from a component-based diagnostic test. This clearly emphasizes the importance of using highly purified and well-characterized allergen preparations for CRD to avoid false positive results. Therefore, an affinity purification protocol was developed using a lysozyme-specific mAb to remove the residual lysozyme from the ion exchange-purified ovomucoid preparation.

The experimental ImmunoCAP test with commercially available ovalbumin was found to contain a considerable amount of ovomucoid as well as some conalbumin. The monomeric forms of ovomucoid and ovalbumin migrated to overlapping positions on SDS-PAGE, and as both are glycoproteins, they were observed as rather diffuse bands. Thus, the presence of ovomucoid in the crude ovalbumin is difficult to detect using SDS-PAGE, which has been reported earlier [12]. Western blotting analysis with ovomucoid- and conalbumin-specific mAbs confirmed the presence of the two allergens in the crude preparation of ovalbumin. The contaminating allergens were removed by specific mAbs in affinity adsorption chromatography. The diffuse band observed at approximately 30–40 kDa in SDS-PAGE of the material eluted from the anti-ovomucoid/anti-conalbumin columns was detected as a faint band in Western blotting with the ovomucoid-specific mAb. However, even if this protein was revealed after mAb-based affinity purification, it cannot be excluded that this may be an unidenti-

fied contaminating protein in the crude ovalbumin preparation.

The crude ovalbumin preparation displayed one dominating peak in SEC analysis. After affinity purification, the purified ovalbumin and the removed ovomucoid could be resolved with overlapping elution profiles verifying that they are difficult to separate according to molecular size, which indeed makes affinity purification an appealing method. It was also indicated from Western blotting that ovalbumin may be present in complex with ovomucoid and possibly also with conalbumin. This may be an explanation for the loss of ovalbumin during the affinity purification, resulting in a protein yield of 66%. However, the presented affinity purification method is an improvement from a previously reported method using successive ion-exchange chromatography and SEC, resulting in a final yield of 16% [14].

Analysis of commercially available conalbumin coupled to ImmunoCAP revealed the presence of ovomucoid and lysozyme. SDS-PAGE and Western blotting with the conalbumin-specific mAb detected a number of aggregated forms in the crude conalbumin preparation. The low amounts of contaminants could only be detected as faint bands in Western blotting under nonreducing conditions with ovomucoid- and lysozyme-specific mAbs, and were removed by affinity adsorption chromatography using the same mAbs. SDS-PAGE and Western blotting of the proteins eluted from the affinity columns verified the presence of both lysozyme and ovomucoid in the crude material.

All aggregated forms of egg-white allergens described in the present study were also observed on SDS-PAGE under reducing conditions (results not shown), suggesting that these aggregates are the result of strong interactions other than disulfide bonds. However, more work is needed before any further conclusions can be drawn about the detailed nature of the aggregates.

To study the prevalence of IgE reactivity to individual egg-white components, CRD using experimental ImmunoCAP tests with highly purified egg-white allergens were performed in 83 egg white-sensitized individuals from northern and southern Europe, the United States and Japan. The specificity of the ImmunoCAP tests was verified by the egg allergen-specific chimeric IgE antibodies. The results showed that IgE reactivity to ovalbumin was most common with a prevalence of 87%, followed in rank by ovomucoid (72%), conalbumin (69%) and lysozyme (58%). Ovalbumin is the main component of egg white comprising more than 50% of the total protein content, which could explain why a majority of egg-sensitized

and egg-allergic patients have IgE reactivity to this allergen as also shown by others [11, 23]. Ovalbumin is a heat-sensitive protein which undergoes conformational changes at elevated temperature [9–10]. This means that the IgE-binding epitopes on ovalbumin might be destroyed after heating, which may be an explanation to why some children sensitive to raw egg white are tolerant to heated egg [8, 24–25]. Formation of the less allergenic S-ovalbumin is a possible explanation to the slightly decreased level of chimeric IgE binding after further purification of ovalbumin. It should be noticed that the chimeric IgE binding is monoclonal and directed to one single epitope on ovalbumin. Normally, IgE responses are polyclonal and less sensitive for conformational changes in allergens.

The rather high prevalence of IgE reactivity to all egg-white allergens in this study is consistent with other studies showing also high prevalence of IgE reactivity to ovomucoid [12, 23, 26], conalbumin [27] and lysozyme [4]. Discrepancies between studies regarding which allergen is actually most important in egg white could be due to methodological differences, patient selection or use of impure allergen reagents. Indeed, significantly more subjects in the present study were positive for IgE (>0.35 kU_A/l) to crude ovomucoid and conalbumin than to the highly purified allergen preparations. The apparent IgE levels were also higher to crude than to highly purified ovomucoid, ovalbumin and conalbumin, a consequence of IgE binding to contaminating allergens.

Measurement of IgE antibodies to individual egg-white components could be of importance in understanding different disease manifestations in egg-allergic patients. For example, several studies have shown that elevated levels of ovomucoid-specific IgE antibodies are associated with allergy to heated egg, while egg-allergic patients with low levels of IgE to ovomucoid may tolerate heated egg and egg in baked foods [8, 11, 28]. IgE to ovomucoid has also been suggested as a prognostic marker for persistent egg allergy [12, 29]. Thus, it is important to use well-characterized and highly purified ovomucoid preparations in the diagnostic test to avoid false high test results. Another example is lysozyme, which is a bacteriostatic enzyme used as a preservative in some pharmaceuticals and foods (for example eye drops and cheeses). Egg-allergic individuals sensitized to lysozyme may therefore react when exposed to such products [30–31]. Moreover, being widely used in the food and pharmaceutical industry, lysozyme is also considered an important occupational allergen, causing asthma via the inhalation route [32–33].

In conclusion, to meet the requirements for accurate CRD we have developed a very sensitive and specific

method for characterization of egg-white allergens based on the use of monoclonal chimeric IgE antibodies. We also present scalable chromatography methods resulting in highly purified preparations of ovomucoid, ovalbumin and conalbumin. Finally, we developed highly specific egg-white allergen component ImmunoCAP tests and showed that IgE reactivity to ovalbumin was most common in egg white-sensitized individuals, followed by ovomucoid, conalbumin and lysozyme.

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