Research Article

The panel of egg allergens, Gal d 1–Gal d 5: Their improved purification and characterization

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Egg proteins represent one of the most important sources evoking food allergic reactions. In order to improve allergy diagnosis, purified and well-characterized proteins are needed. Although the egg white allergens Gal d 1, 2, 3 and 4 (ovomucoid, ovalbumin, ovotransferrin, and lysozyme) are commercially available, these preparations contain impurities, which affect exact *in vitro* diagnosis. The aim of the present study was to set up further purification protocols and to extend the characterization of the physicochemical and immunological properties of the final batches. The egg white allergens Gal d 1–4 were purified from commercial preparations, whereas Gal d 5 (α-livetin) was purified from egg yolk. The final batches of Gal d 1–5 consisted of a range of isoforms with defined tertiary structure. In addition, the IgE binding capacity of the purified egg allergens was tested using allergic patients' sera. The allergen batches will be further used to set up allergen specific diagnostic assays and to screen a larger collection of patients' sera.

Keywords: Allergens / Egg / NMR / p*I* / Purification

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1 Introduction

1.1 Prevalence of egg allergy

Egg and milk are among the most frequent causes of allergic reactions in children [1], but estimations of the total prevalence of allergy to egg vary considerably. Eggesbø *et al*. [2] estimated the prevalence in Norwegian children aged 2½ years to 1.6%, while the corresponding figure for German preschool children (5–6 years of age) was found by Schäfer *et al*. [3] to be 2.8%. The allergenicity of egg white is reduced by cooking [4] or other denaturation [5, 6].

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Abbreviations: CD, circular dichroism; **CSA,** chicken serum albumin; **SEC,** size exclusion chromatography

The dominating allergens from egg are the four egg white proteins ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3) and lysozyme (Gal d 4) [4, 7]. These are also the most abundant proteins in egg white, representing 11, 54, 12, and 3.4%, respectively, of the egg white proteins [8]. In the yolk, the protein α-livetin (Gal d 5) is of interest because it is involved in the bird-egg syndrome [9, 10], where sensitization is supposed to occur by inhalation during contact to birds. The prevalence of the bird-egg syndrome is uncertain, but it is less common than food allergy to egg and more widespread in adults than in children [9, 10]. α-Livetin is identical to [11] or very closely related to chicken serum albumin (CSA) and is partly heat resistant. Chicken meat allergy is rare [1, 6]; it may be related to the bird-egg syndrome [12]. In addition to Gal d 1–5, a large range of proteins from egg white [1] and egg yolk [4] have been shown to bind IgE from egg allergic patients, but with lower prevalence.

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The starting material for the present purification of egg white proteins was partly purified commercial preparations. The need to improve purification of the commercial preparations is well-documented [7, 13]. The starting material for α-livetin was egg yolk, not chicken serum, as it has not been unambiguously demonstrated that the two proteins are identical [11].

Gal d 1–3 and 5 exist in several isoforms, which are all able to induce an allergic reaction. It has therefore been considered important that the final purified allergen preparations contain a representative spectrum of the isoforms present in the egg. Many of the isoforms are differently charged, and for each preparation it was ensured that all isoforms detected by IEF in egg were present. This was not part of the characterizations of the formerly presented purification procedures for egg proteins, neither was the folding of the proteins in the preparations [7, 13].

1.2 Egg allergens

1.2.1 Ovomucoid, Gal d 1

Ovomucoid consists of 186 amino acid residues and 25% carbohydrate [14], as determined by MS. It is very stable under *in vivo* and *in vitro* conditions being a serine protease inhibitor with 9 disulfide bonds and no free –SH groups [15]. The carbohydrate chains are penta-antennary, heterogeneous and partially sialylated [16, 17], resulting in substantial mass and charge heterogeneity of native ovomucoid. The carbohydrate chains seem to be unique and have not been reported to cause carbohydrate-based cross-reactivity.

1.2.2 Ovalbumin, Gal d 2

Ovalbumin consists of 385 amino acid residues [18] and 3% carbohydrate (by weight). It has one disulfide bond and four free –SH groups, which result in some dimerization. Native ovalbumin displays considerable charge heterogeneity because of sequence variations, and phosphorylation in two sites with a reported ratio of 1:2:8 of zero, one and two phosphate groups, respectively [19]. Finally, during storage in atmospheric air, ovalbumin rearranges to *S*-albumin, a conformationally different form, exposing an additional carboxylate group [20]. In total, the post-translational modifications increase the sequence-derived M_r of 42 750 to 44 000–45 000.

1.2.3 Ovotransferrin, Gal d 3

Ovotransferrin, also called conalbumin, consists of 686 amino acids with 15 disulfide bonds and no free –SH groups [21]. It contains 3% (by weight) of carbohydrate. Charge heterogeneity arises from sequence variations and variations of Fe^{3+}. Ovotransferrin can bind two Fe^{3+} in association with binding of an anion [22], resulting in one extra negative charge *per* bound ferric ion. Ovotransferrin in egg white is normally without ferric ions. The theoretical p*I* of the dominating form is 6.69, and the M_r is 75 828. The M_r of glycosylated ovotransferrin is approx. 77 000.

1.2.4 Lysozyme, Gal d 4

Lysozyme consists of 129 amino acid residues, with four disulfide bonds and no free –SH groups [23]. Lysozyme has no post-translational modifications and is homogeneous with a theoretical p*I* of 9.3e, and an M_r of 14 313.

1.2.5 α-Livetin, Gal d 5

α-Livetin is, like CSA, designated Gal d 5. CSA consists of 589 amino acid residues (Swiss-Prot entry P19121). It is homologous to mammalian serum albumins (47 and 44% identity to human and bovine SAs, respectively). The protein has one potential glycosylation site and 35 cysteine residues. Based on similarity with other serum albumins, the –SH groups are expected to be linked in 17 disulfide bridges leaving one free –SH group giving rise to dimerization. The theoretical M_r of α-livetin is 66 815 and the theoretical p*I* 5.31.

2 Materials and methods

2.1 Chemicals

Tris (Trizma base) was purchased from Sigma Chemical (St. Louis, MO, USA). CBB G250 was from Merck (Darmstadt, Germany). All other chemicals were of analytical grade. Proteins used for calibration of size exclusion chromatography (SEC) were aprotinin, myoglobin, β-lactoglobulin and BSA, all from Sigma Chemical. Water was drawn from a Milli-Q System equipped with an Organex-Q cartridge from Millipore (Bedford, MA, USA). Dialysis was performed at 4°C. If not stated otherwise, procedures were performed at 20°C.

2.2 Proteins

Preparations of ovomucoid (T2011, lot 20h8170), ovalbumin (A5503, lot 71k7028), ovotransferrin (C0755, lot 011k7052) and lysozyme (L6876, lot 57h7045) were purchased from Sigma Chemical. Egg yolk was obtained from eggs from ordinary retailers stamped from the producer "Hedegården," Denmark. Egg white extract was prepared according to Langeland [24].

2.3 Sera

Sera from patients ($n = 10$) with established egg allergy according to convincing case history and positive radioallergosorbent test values were identified and stored at –20°C until use. For immunoblots, the sera were pooled, and in the ELISA, single serum testing was performed. For control experiments, sera from nonatopic persons ($n = 3$) were used in parallel.

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2.4 Purification procedures

2.4.1 Ovomucoid, Gal d 1

Ovomucoid was purified according to Ebbehøj *et al*. [13] by anion exchange chromatography on Q-Sepharose FF (XK 16/20, Pharmacia, Uppsala, Sweden) in 20 mM triethanolamine (TEA), pH 7.3, and eluted with 295 mL of linear gradient steps from 0 to 100% 1 M NaCl (15 mL 0%, 55 mL to 25%, 190 mL to 65%, 35 mL to 100%).

After freeze drying, it was subjected to SEC on Sephacryl S 200 (Pharmacia; 1.6×95 cm^2) in 200 mM NH_4HCO_3, elution rate 3 cm/h. Pure ovomucoid fractions were dialyzed toward 2×50 volumes of H_2O and freeze dried.

2.4.2 Ovalbumin, Gal d 2

The purification of ovalbumin was modified from Ebbehøj *et al*. [13] by anion exchange chromatography on Q-Sepharose FF (XK 16/20, Pharmacia) in 20 mM TEA, pH 7.2, and eluted with 140 mL of a gradient from 0 to 40% of 1 M NaCl. Selected fractions were dialyzed against 2×50 volumes of H_2O, freeze dried, and submitted to cation exchange chromatography on SP Sepharose FF (Pharmacia; 1×8.5 cm^2) in 20 mM HCOONa, pH 4.2, and eluted with 100 mL of a linear gradient of NaCl from 0 to 1 M. Selected fractions were freeze dried, dialyzed against 20 mM Tris-HCl, pH 8.0, and submitted to anion exchange chromatography on Q-Sepharose FF (XK 16/20, Pharmacia) in Tris-HCl, pH 8.0, with 66 mL of a stepwise gradient from 0 to 1 M NaCl (6 mL 0%, 44 mL to 8%, 8 mL to 50%, 8 mL to 100%). Selected fractions were freeze dried and submitted to SEC on Sephacryl S 200 (Pharmacia; 1.6×95 cm^2) in 200 mM NH_4HCO_3, elution rate 3 cm/h. Selected fractions were dialyzed toward 2×50 volumes of 5 mM NaH_2PO_4, pH 7.2, and freeze dried.

2.4.3 Ovotransferrin, Gal d 3

Ovotransferrin was purified by SEC on a Superdex 75 (Hiload 26/60, AP Biotech, Sweden) in 200 mM NaAc, pH 4.0, at 4°C, elution rate 2.3 cm/h.

Selected fractions were dialyzed against 50 mM Tris-HCl, pH 8.7, and submitted to anion exchange chromatography on Q-Sepharose FF (XK 16/20; Pharmacia) in 50 mM Tris-HCl, pH 8.7, with 300 mL of a linear gradient from 0 to 0.5 M NaCl. Selected fractions were dialyzed against 2×50 volumes of H_2O and freeze dried.

2.4.4 Lysozyme, Gal d 4

Lysozyme was purified according to Ebbehøj *et al*. [13] by cation exchange chromatography on SP Sepharose (Pharmacia; 1×8.5 cm^2) in 20 mM Bicine, pH 8.7, with 200 mL of a stepwise gradient from 0 to 0.2 M NaCl (63 mL 0%, 57 mL to 75%, 80 mL to 100%). Selected fractions were dialyzed against 2×50 volumes of H_2O and freeze dried.

2.4.5 α-Livetin, Gal d 5

α-Livetin was isolated from yolk from hen's egg. A protocol was developed based on the method presented by Burley [25], starting with a single hen's egg. Twenty milliliters were mixed with one volume of 0.16 M NaCl and ultracentrifuged at $100\,000 \times g$ at 10°C for 30 min to remove the granula. The granula-free solution was mixed with one volume of 4 M NaCl and ultracentrifuged at $100\,000 \times g$ for 20 h at 10°C (Beckman L8 7 M Ultracentrifuge). After removal of the upper layer, the supernatant was dialyzed against 50 mM CH_3COONH_4, pH 6.8, centrifuged, filtered through a 45 µm acetate filter (GE Osmonic, Minnetoka, USA) and submitted to anion exchange chromatography (DEAE-Sepharose CL 6B; Pharmacia; 1.6×16 cm^2) with a linear gradient of 170 mL CH_3COONH_4 from 50 mM to 0.4 M, followed by 50 mL of 1 M NaCl. Fractions containing α-livetin were dialyzed against 50 mM Tris-HCl, pH 8.5. Subsequently, anion exchange chromatography on a Q-Sepharose FF column (XK 16/20, Pharmacia) was performed in 50 mM Tris-Cl, pH 8.7, with a linear gradient of 150 mL from 0 to 0.5 M NaCl. Fractions containing α-livetin were selected and freeze dried and submitted to gel filtration on Superdex 75 at 4°C (Hiload 26/60, AP Biotech) in 200 mM CH_3COONa, pH 4.0, at 4°C with an elution rate of 2.3 cm/h. Selected fractions were dialyzed against 2×50 volumes of H_2O and freeze dried.

2.5 Electrophoresis and immunoblotting

SDS-PAGE was run in NuPAGE 10% Bis-Tris gels from Invitrogen (Carlsbad, CA, USA) in MOPS or 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer. Standard (Mark 12, Invitrogen) and samples were reduced with DTT. Gels were fixed and stained with CBB G250 according to [26], modified by replacement of methanol with ethanol.

For immunoblots, purified egg allergens (1.5 µg *per* lane) and egg protein extract (4 µg *per* lane), respectively, were separated by SDS-PAGE and blotted to nitrocellulose membrane. After blocking with BSA, the membranes were incubated with appropriately diluted serum pool (serum 1–10) from egg allergic patients. Bound IgE was detected using ^{125}I-labeled antihuman IgE (MALT Allergy System Isotope Reagent, IBL Hamburg, Germany). For controls, normal human serum samples were used, as well as a buffer incubation step omitting the first antibody.

IEF was run in 5% polyacrylamide NOVEX gels, 1 mm, pH 3–7 from Invitrogen, according to the protocol provided by the manufacturer, on a Novex Mini-Cell, X-Cell SureLock™ Electrophoresis Cell (Invitrogen) placed in an ice bath. Proteins in the gels were fixed in 3.5% w/v sulfosalicylic acid, 12% trichloroacetic acid w/v and then stained as SDS-PAGE gels. Staining for protease inhibitor activity was performed according to Hejgaard [27]. Two standards were used: Amersham's broad p*I* range standard (Amersham) and Serva IEF Markers 3–10, (Serva Electrophore-

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sis, Heidelberg, Germany). The identity of bands in the p*I* markers were confirmed by means of MALDI-TOF MS.

2.6 Amino acid analysis and protein sequencing

Amino acid analysis was performed according to Barkholt and Jensen after hydrolysis for 20 h in hydrochloric acid [28]. All quantification was done by amino acid analysis. N-terminal sequencing was performed on an Applied Biosystems Procise 494 Sequencer (Applied Biosystems, Foster City, CA, USA) in the liquid phase mode. Cysteine residues were not modified.

2.7 MS

Sequence analysis of ovomucoid, ovotransferrin and α-livetin was performed by LC-MS-MS after an in-solution tryptic digest of the purified protein, using the Proteoextract trypsin digestion kit (Calbiochem, San Diego, USA). Resulting peptides were separated by capillary RP-HPLC (Waters, Milford, USA) directly coupled to the mass spectrometer *via* the Waters Nanoflow spray head (precolumn: Waters Nanoease Symmetry 300 trap column; separating column: Waters Nanoease Atlantis dC18, connected *via* a 10 port stream select valve). The flow rate was adjusted to 300 nL/min by T-splitting. Peptides were eluted with an ACN gradient (solvent A 0.1% v/v formic acid/5% v/v ACN, solvent B 0.1% v/v formic acid/95% v/v ACN; 5–45% B in 90 min). For sequence identification, a combined Swiss-Prot/Trembl database was used.

Sequence analysis of ovalbumin and lysozyme in solution and IEF-standards in gels were performed by Bruker MALDI TOF–TOF Ultraflex II (BrukerDaltonics, Bremen, Germany) after tryptic cleavage [29]. For the analyses of the spectra, Bruker Daltonics FlexControl (3.0) was applied, and for the search in the MASCOT Server (www.matrixscience.com), Bruker Daltonics BioTools (3.1) was used. All identifications presented were significant ($p < 0.05$).

2.8 Circular dichroism (CD)

Far UV CD spectra of purified egg allergens were recorded with a JASCO J-810 spectropolarimeter (Jasco, Essex, UK), at 20°C in aqueous solutions. Protein samples (0.1 μg/mL) were measured in quartz cuvettes (Hellma, Mullheim, Baden, Germany) of 0.1 and 0.2 cm path lengths, respectively. All spectra were corrected for the proper baseline using the corresponding aqueous solution. Absorption between 190 and 260 nm was monitored at 0.5 nm intervals. The obtained spectra represent an average of three consecutive scans. Results of each sample were averaged, and the mean residue ellipticity (θ) was expressed as deg cm^2 $dmol^{-2}$.

2.9 NMR analysis

NMR analyses were performed according to the following protocol: Before and after the NMR experiments, the allergens were stored at −20°C. A solution of each allergen was prepared in 0.45 mL H_2O plus 0.05 mL of D_2O. The concentrations were 0.18 mM for ovomucoid, 0.14 mM for ovalbumin, 0.08 mM for ovotransferrin, 0.43 mM for lysozyme and 0.09 mM for α-livetin. The solutions were put in high-quality NMR tubes with Ar as head-space gas. Two High Resolution NMR experiments were carried out, by means of a Bruker Avance 700 spectrometer operating at a proton resonance frequency of 700 MHz (11.7 Tesla), at 25°C. The two experiments are different in the method of managing the water signal: the zgpr experiment (spectra not reported) minimizes it, while the zgesgp experiment suppresses the water peak. For each experiment, 256 scans were programmed for ovomucoid, 512 scans for ovalbumin, 1228 scans for ovotransferrin, 921 scans for lysozyme and 7168 scans for α-livetin.

2.10 IgE ELISA

IgE binding ability of purified egg allergens was tested in a fluid phase ELISA. One microgram of each of the purified allergens was adsorbed onto wells of microtiter plates (Nunc Maxisorp, Nunc International, Roskilde, Denmark) overnight at 4°C. After blocking with Tris-buffered saline containing 0.5% v/v Tween-20 and 3% w/v nonfat dry milk (at room temperature, for 2 h), 1:4 diluted sera were applied onto the plates and incubated overnight at 4°C. After washing, the plates were incubated with a 1:1000 diluted alkaline phosphatase-conjugated mouse antihuman IgE antibody (BD Pharmingen, San Diego, CA, USA) for 2 h at room temperature. Color development was obtained using disodium *p*-nitrophenyl phosphate substrate tablets (Sigma–Aldrich, Steinheim, Germany), and OD was measured at 405 nm. Buffer and three sera of nonallergic subjects were used as negative controls. OD values were counted positive if they exceeded the mean OD of the negative controls by more than three SDs.

2.11 Determination of LPS

The starting material for egg white proteins and the five purified allergens were tested for bacterium-derived endotoxin levels in a limulus amoebocyte lysate assay, according to the manufacturer's instructions (Bio Whittaker, Verviers, Belgium).

3 Results and discussion

3.1 Physicochemical characterization of Gal d 1–5 and evaluation of purity

For all the obtained preparations, the amino acid composition was in accordance with the literature and the values expected after 20 h hydrolysis [28], giving a first indication of the authenticity of the purified proteins. The existence of

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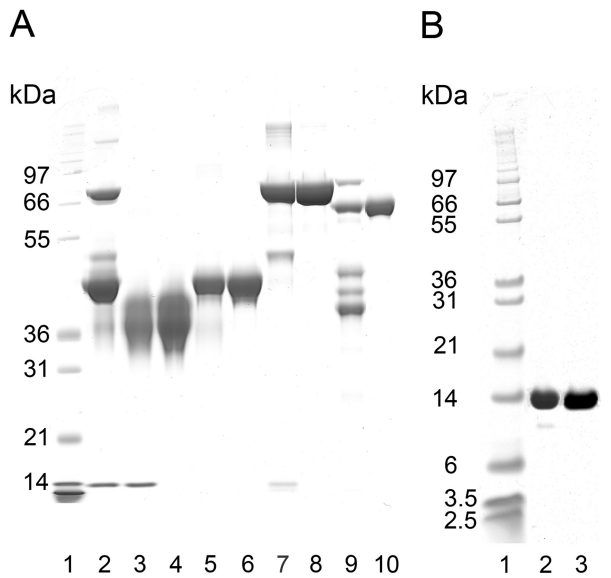

Figure 1. SDS-PAGE of egg allergens before and after purification. Coomassie stained SDS-PAGE. (A) Novex Nupage 10% Bis-Tris, MOPS buffer. Standard Mark 12, 5 μg (lane 1). Egg white extract, 20 μg (lane 2). Ovomucoid before and after purification, 5 μg (lanes 3 and 4). Ovalbumin before and after purification, 3 μg (lanes 5 and 6). Ovotransferrin before and after purification 3 μg (lanes 7 and 8). Egg yolk extract, 20 μg (lane 9). α-Livetin, 3 μg (lane 10). (B) Novex Nupage 10% Bis-Tris, MES buffer. Standard Mark 12, 5 μg (lane 1). Lysozyme before and after purification, 5 μg (lanes 2 and 3).

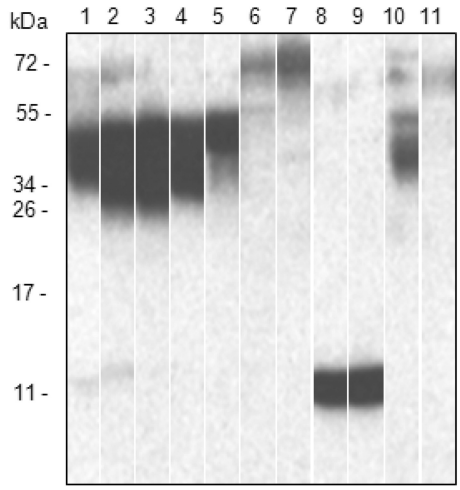

Figure 2. Immunoblot analysis of egg allergens before and after purification. IgE binding was tested to egg white protein extract (lane 1), Gal d 1 before and after purification (lanes 2 and 3), Gal d 2 before and after purification (lanes 4 and 5), Gal d 3 before and after purification (lanes 6 and 7), Gal d 4 before and after purification (lanes 8 and 9), egg yolk protein extract (lane 10), and purified Gal d 5 (lane 11). After electrophoresis, proteins were transferred to nitrocellulose sheets and incubated with a serum pool of allergic patients sera (serum 1–10). Bound IgE antibodies were detected by ^{125}I-labeled anti IgE antibodies.

several isoforms of each allergen (except lysozyme) prevents the analysis of purity by chromatography, whether ion exchange chromatography (IEC) or RP, as some isoforms would appear as impurities. Therefore, the degrees of purity were evaluated from overloaded Coomassie stained gels (results not shown).

3.1.1 Ovomucoid, Gal d 1

From SDS-PAGE (Fig. 1A), it appears that the starting material was contaminated with lysozyme (Gal d 4) and ovotransferrin (Gal d 3). Both contaminations bound IgE, as seen in Fig. 2. The final product was judged to be >99% pure. The range of apparent M_r values (34 000–40 000) is in accordance with the expected heterogeneity and the bias of glycoproteins toward higher apparent M_r values. SEC of purified ovomucoid showed one peak, corresponding to an apparent M_r of 46 000 (data not shown). As ovomucoid is easily washed out during Coomassie staining, the IEF gels were stained for protease inhibitor activity (Fig. 3B). Four distinct bands ranging from p*I* 3.7 to 4.5 were seen. The N-terminal amino acid sequence was AEVDXSRFPN-, which is in accordance with the literature [15] (residue 5 is Cys). MS analysis confirmed the identity as ovomucoid. The final yield was 71% of the starting material.

3.1.2 Ovalbumin, Gal d 2

From SDS-PAGE (Fig. 1A), it appears that the starting material was contaminated with ovomucoid (Gal d 1) and other components. Immunoblot (Fig. 2) also revealed ovomucoid as an IgE binding contaminating component.

SEC showed one major peak corresponding to the apparent M_r 46 000, and a small peak representing 4% of the absorbance at 280 nm at an M_r representing the dimer of ovalbumin (data not shown). SDS-PAGE (Fig. 1A) of the purified ovalbumin gave an apparent M_r of 43 000–46 000, with a distinct band below the major band. The upper band eluted in the first part of the ovalbumin fractions by anion exchange chromatography, whereas the material giving rise to the lower band was displaced toward the later fractions. Therefore, the lower band is supposed to be *S*-ovalbumin, which has a lower p*I* and has been shown to fold differently from native ovalbumin, even after denaturation [20]. The preparation was judged to be >99% pure. By IEF (Fig. 3A), four distinct bands were detected between 4.6 and 4.8, representing the native ovalbumins and *S*-ovalbumins with various phosphorylations. The same bands were seen in egg white, although in different ratios (Fig. 3A). Desert *et al.* [30] observed three bands between p*I* 4.6 and 4.8. N-terminal sequencing gave no significant result in accordance with the expected acetylation of the N-terminal residue of ovalbumin [18]. However, MS analysis confirmed the iden-

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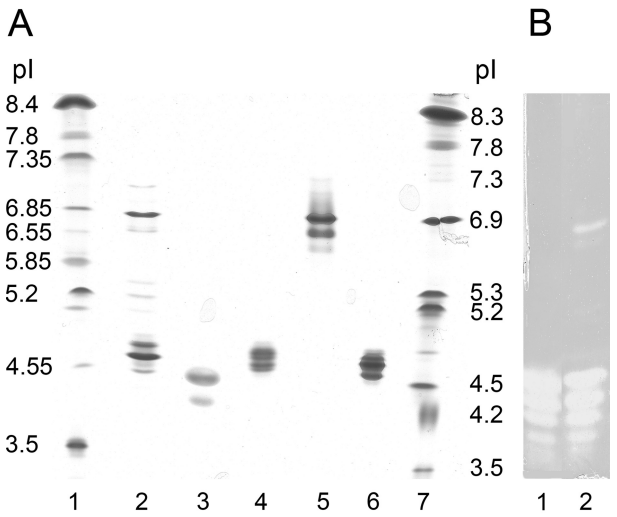

Figure 3. IEF of the purified allergens (IEF pH 3–7). The p*I*-standards are common for (A) and (B). (A) Coomassie stained. Standard, Amersham, p*I* 3–10 (lane 1). Egg white, 20 μg (lane 2). Ovomucoid, 10 μg (lane 3). Ovalbumin, 4 μg (lane 4). Ovotransferrin, 5 μg (lane 5). Alpha-livetin, 5 μg (lane 6): Standard, Serva, p*I* 3–10 (lane 7). (B) Stained for visualization of protease inhibitors. Egg white, 80 μg (lane 1). Ovomucoid, 14 μg (lane 2).

tity as ovalbumin. The final yield was 16% of the starting material.

3.1.3 Ovotransferrin, Gal d 3

Ovotransferrin turned out to be difficult to purify from the lots available, whether the starting material was ferric-containing or nonferric-containing (T. Have, unpublished results). Both products contained some material of higher M_r than ovotransferrin and a long range of material of lower M_r (Fig. 1A). After anion chromatography at pH 7.2 and 8.7, both products had a strong tendency to break down into smaller fragments during dialysis and freeze drying, possibly due to the presence of one ore more proteases. Addition of the covalent binding serine protease inhibitor phenylmethylsulfonyl fluoride did not change this. Therefore, SEC at pH 4.0 and 4°C was chosen as the first purification step. This step removed some high M_r material, which was difficult to get rid of by IEC and which may have contained the protease activity, as the stability of ovotransferrin in the selected fractions was much improved. The iron-free product was chosen as starting material, but apparently ferric ions had been added during production [31] and incompletely removed, as seen by the weakly red color of the product.

The initial purification step, SEC on a Superdex 75 column, resulted in one major asymmetric peak corresponding to M_r 76 000, from which the material for further purification was selected by SDS-PAGE (data not shown). In SDS-PAGE (Fig. 1A), the apparent M_r of the purified ovotransferrin was 78 000 and the purity was estimated to be >95%. Since all additional bands corresponded to lower molecular masses, and several analyzed bands had the N-terminal sequence of ovotransferrin, it was concluded to be degradation products of ovotransferrin. The p*I* values of the components in the purified ovotransferrin were those found in egg white, although the ratio between the different forms was different. IEF resulted in three distinct bands (Fig. 3A) corresponding to p*I* 6.4, 6.6 and 6.9. The higher p*I* value is supposed to correspond to the iron-free ovotransferrin. It is the dominating form in both egg white and purified ovotransferrin, but the purified product contains more of the lower p*I* bands than seen in egg white because of the addition of ferric ions during purification. Desert *et al*. [30] found the p*I* values 6.4, 6.6 and 6.8 for ovotransferrin containing 2, 1 and no ferric ions, respectively. The N-terminal amino acid sequence was APPKSVIRWX-, in accordance with the literature [21] (residue 10 is Cys). MS analysis confirmed the identity as ovotransferrin. The yield was 9% of the starting material.

3.1.4 Lysozyme, Gal d 4

SDS-PAGE (Fig. 1B) of the starting material revealed an unidentified contaminating component with apparent M_r of 11 000. The final preparation was >99% pure. Lysozyme with a theoretical p*I* 9.3 was not seen in pH 3–10 IEF gels, neither in the lane with egg white nor with the purified protein. The N-terminal amino acid sequence was determined as KVFGRXELAA-, which is in accordance with the literature (residue 6 is Cys) [23]. MS analysis confirmed the identity as lysozyme. The yield was 65% of the starting material.

3.1.5 α-Livetin, Gal d 5

An apparent M_r of 66 000 was found for α-livetin by SDS-PAGE (Fig. 1A), with an estimated purity of >98%. SEC showed one peak corresponding to an apparent M_r of 66 000 and 10% dimer. By IEF, four bands between p*I* 4.6 and 4.8 were seen (Fig. 3A). The isoforms in the preparation could not be compared to the isoforms in yolk, as yolk contains numerous proteins, making identification of α-livetin by IEF difficult. The N-terminal amino acid sequence DAEHKSEIAH- is the N-terminal sequence of CSA presented by Rosen and Geller [32]. MS analysis (71% sequence coverage) was in accordance with the sequence of CSA (Swiss-Prot entry P19121). The content of α-livetin in yolk is reported to be 0.6% w/w [31]. The obtained yield corresponds to 10% of that.

3.2 Folding of Gal d 1–5

An estimation of the folding topology of the purified proteins was obtained by CD that either clearly showed (ovomucoid and lysozyme) or provided evidence (ovalbumin, ovotransferrin and α-livetin) that the proteins were folded

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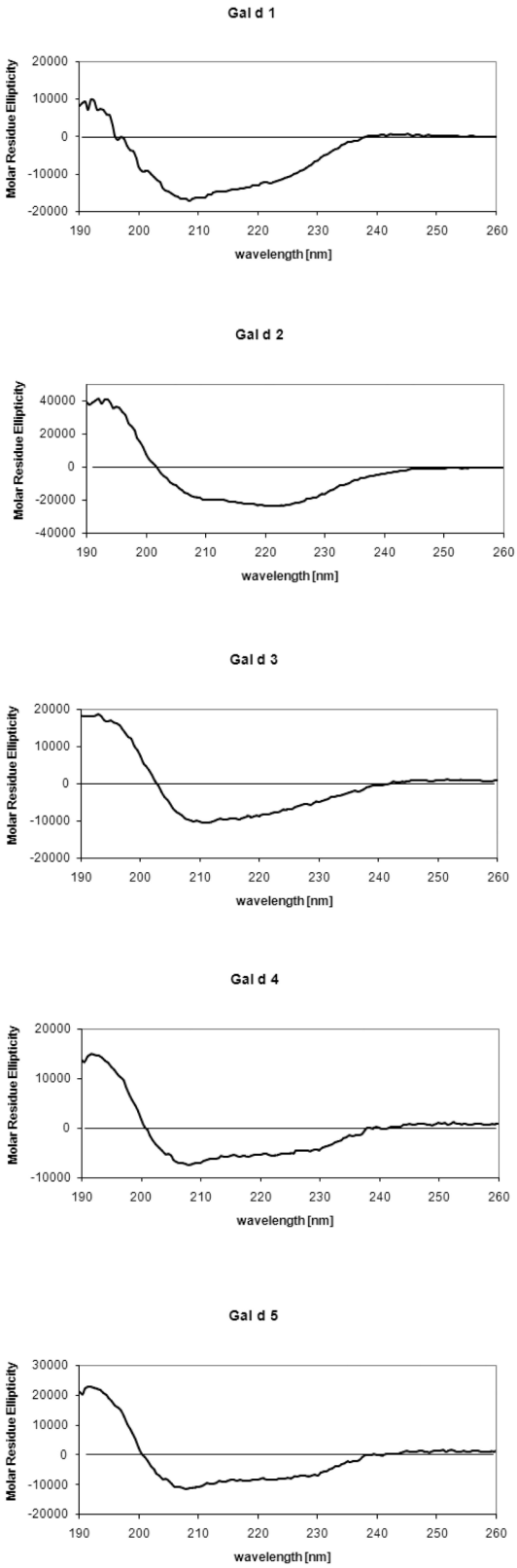

Figure 4. CD spectra of the purified egg allergens Gal d 1 (ovomucoid), Gal d 2 (ovalbumin), Gal d 3 (ovotransferrin), Gal d 4 (Lysozyme) and Gal d 5 (α-livetin).

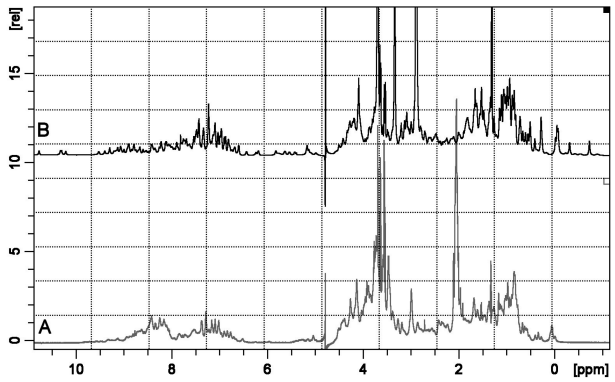

Figure 5. NMR spectra of ovomucoid and lysozyme. (A) ^{1}H 700 MHz spectrum of ovomucoid (33.0 kDa), zgesgp experiment, 256 scans, 25°C. (B) ^{1}H 700 MHz spectrum of lysozyme (14.0 kDa), zgesgp experiment, 1228 scans, 25°C.

and contained α-helical and β-sheet structures (Fig. 4). For ovalbumin, lysozyme and α-livetin, the α-helical structures are predominant according to the CD spectra.

NMR spectroscopy was used to determine the presence and extent of tertiary structure. As for ovomucoid, NMR analysis resulted in signals dispersed in the whole spectral window, from −0.1 to 9.9 ppm, indicating the presence of a rigid tertiary structure, accompanied by several peaks that were often not resolved near the baseline (Fig. 5A). The dispersion of aromatic and amide protons, of α-protons and of methyl peaks is a reliable index of extended and rigid tertiary structure, although portions of the protein seem mobile and flexible. This is consistent with the structural information available about ovomucoid from different species. Presence of carbohydrate could be noticed in the 3.0–4.0 ppm region.

The NMR spectra of ovalbumin were partially unresolved due to its high molecular mass (Fig. 6A). Nevertheless, the signature of its tertiary structure is unquestionable with dispersed NH-, aromatic, H-α- and methyl peaks in the whole range from −0.4 to 9.8 ppm.

The equivalent studies of ovotransferrin were heavily affected by the high molecular mass of this allergen, but signals indicating folding are present in the whole spectral window, and an extended rigid tertiary structure can be deduced (Fig. 6B). On the other hand, the presence of flexible and mobile zones cannot be excluded because the single peaks are basically unresolved. These findings are consistent with the structural information provided by the literature [33].

The tertiary structure of lysozyme is apparent from the NMR spectra featuring narrow and well-separated peaks (Fig. 5B).

The partial resolution of the NMR spectra of α-livetin (Fig. 6C) was due to its large molecular mass. Anyway, it must be noted that there is a peak at −0.6 ppm, followed by unresolved signals upfield, and there is a peak at 10.2 ppm,

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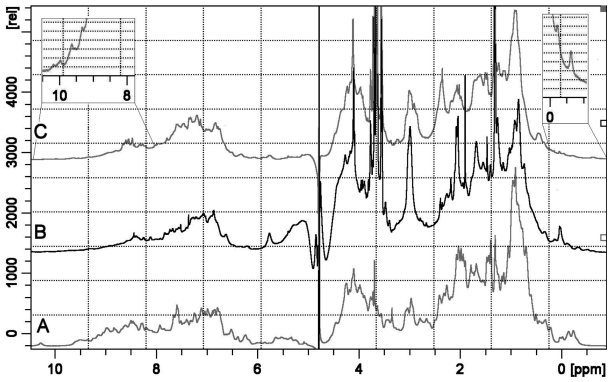

Figure 6. NMR spectra. (A) ^{1}H 700 MHz spectrum of ovalbumin (44.0 kDa), zgesgp experiment, 512 scans, 25°C. (B) ^{1}H 700 MHz spectrum of ovotransferrin (78.0 kDa), zgesgp experiment, 1228 scans, 25°C. (C) ^{1}H 700 MHz spectrum of α-livetin (69.0 kDa), zgesgp experiment, 7168 scans, 25°C.

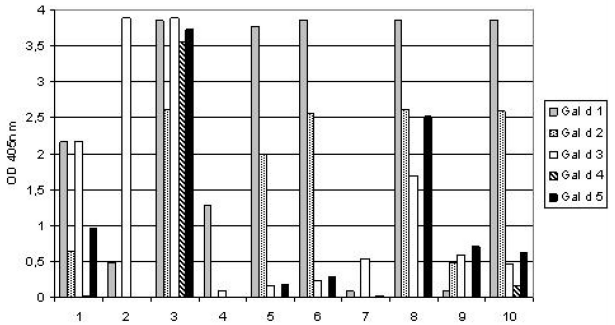

Figure 7. IgE ELISA of purified Gal d 1–5. Ten sera from egg allergic patients were tested for IgE binding to purified Gal d 1–5. In parallel, normal human serum samples were tested (negative controls). Values are expressed in OD after subtraction of OD values obtained from negative controls plus 3 × SD.

grouped with other signals upfield. Furthermore, there are partially resolved peaks around the water signal which may be assigned to α-protons. Therefore, the protein has a rigid and extended tertiary structure, and probably, in addition flexible and mobile parts. The sharp peaks in the 3.5–3.8 range could be due to glycosylation.

3.3 Antigenicity/allergenicity of Gal d 1–5

In fluid phase assay (ELISA), 10 sera from egg allergic patients were tested with purified Gal d 1–5 (Fig. 7). All the sera tested (10/10) displayed specific IgE binding to Gal d 1 and Gal d 3. Gal d 2 was recognized by seven out of ten sera and Gal d 5 by eight sera. Only three sera had specific IgE raised against Gal d 4. In summary, the purification procedures outlined here resulted in preparations of allergens which maintained their IgE binding activity throughout the purification procedures. IgE binding of the serum pool to the starting material, commercial fractions of Gal d 1–4, the purified batches as well as to egg white, egg yolk extract and purified Gal d 5 were tested in immunoblots (Fig. 2). Purification of Gal d 1 resulted in a loss of additional IgE reactive bands corresponding to Gal d 3 and Gal d 4 present in the starting material (Fig. 2, lanes 2 and 3). Purification of Gal d 2 resulted in a loss of additional IgE binding to Gal d 1 (Fig. 2, lanes 4 and 5). Gal d 3 starting material displayed no additional IgE binding component when compared to the final purified protein fraction (Fig. 2, lanes 6 and 7), as well as Gal d 4 had no additional IgE binding component as judged by immunoblot (Fig. 2, lanes 8 and 9). Gal d 5, purified from egg yolk, resulted in a single IgE binding component as seen in immunoblot (Fig. 2, lanes 10 and 11). In addition, controls with normal human serum samples and buffer were performed, which tested negative in all cases (data not shown).

In summary, the purification procedures outlined here resulted in preparations of allergens which maintained their IgE binding activity throughout the purification procedures and with no detectable IgE binding contaminants.

3.4 Endotoxins

The contents of endotoxins in starting materials as well as the purified allergens were very low, ranging from no detectable endotoxins (Gal d 1) present to 72 endotoxin units *per* mg (Gal d 3).

4 Concluding remarks

There is a considerable discrepancy between the p*I* values for egg proteins found in the literature, but the results presented here are generally in agreement with the results of Desert *et al*. [30]. The four distinct bands seen for ovomucoid are remarkable, because the published sialylated carbohydrate structures [17] seem to present the possibility of a continuous range of p*I*s. Desert *et al*. [30] found bands in the region 4.0–4.5, but unresolved. Although the ratios between the detectable isoforms for each allergen were not identical to the ratio in egg white, all forms appeared to be represented in the preparations, as required.

For α-livetin, the four bands seen in IEF suggest sequence heterogeneity or post-translational modifications in accordance with the NMR results indicating glycosylation, even though no residues with modifications were identified by MS. The sequence presents one potential *N*-glycosylation site (Swiss-Prot entry P19121). No information on post-translational modifications of CSA has been published. The above results support the claim of α-livetin being identical to CSA. However, final proof covering the full length protein is still lacking.

The purified preparations obtained in the present study contained the whole spectrum of isoforms found in egg, as judged from p*I* values determined with IEF and apparent M_r

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range determined by SDS-PAGE and SEC. The folding conformation of all five preparations was considered satisfactory, as monitored by CD and NMR. Despite indications that parts of the purified allergen preparations might be present in the unfolded state, the quality obtained for the proteins with regard to correct folding was considered sufficient for the egg allergen panel. Finally, the preparations of the individual egg proteins bound IgE from all relevant sera tested, lending further support to the authenticity of the purified products to be used for allergen specific *in vitro* diagnosis of egg allergy.

In conclusion, compared to formerly described egg allergen panels, the characterization of the present was improved by the demonstration of folded protein and evidence for coverage of the complete ranges of isoallergens. This panel of well-characterized egg allergens will be further used to screen a significant number of egg allergic patients' sera within the EC-funded project Europrevall. This in turn will provide actual data on the prevalence of egg allergy in Europe, as well as give better insight into the molecular basis for potential cross-reactivity.

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The authors have declared no conflict of interest.

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