

Allergenicity of Hen's Egg-white Proteins: IgE Binding of Native and Deglycosylated Ovomucoid

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Ovomucoid (OM) is a major allergen of hen's egg-white. Carbohydrate moieties from the glycoprotein were removed by chemical deglycosylation. Deglycosylated ovomucoid (d-OM) consists of five isoforms with molecular weights (MW) between 20.7 and 21.5 kDa whereas native OM has a MW of 28 kDa as determined by matrix-assisted laser desorption/ionization time of flight mass spectrometry. The IgE-binding properties of both proteins were investigated. All patients' sera tested, showed strong IgE binding to both native OM and d-OM in SDS-PAGE/immunoblot. In enzyme-allergosorbent test experiments, 50% inhibition of IgE binding was observed, at almost the same inhibitor concentrations of 67 and 57 ng ml⁻¹ for native OM and d-OM respectively. Results indicate that only epitopes on the protein backbone are responsible for IgE binding while carbohydrate residues do not participate in allergenic structures of OM.

Keywords: Food allergy, egg-white, ovomucoid, deglycosylation, IgE binding, SDS-PAGE/immunoblot, EAST inhibition, MALDI-TOF, CZE

INTRODUCTION

Hen's eggs are one of the most frequent causes of adverse reactions to food in children (Bernhisel-Broadbent *et al.*, 1994; Crespo *et al.*, 1995) and adults (Nørgaard & Bindslev-Jensen, 1992; Wüthrich, 1993). Symptoms are often due to accidental ingestion of hidden food allergens (Yman *et al.*, 1994; Moneret-Vautrin & Kanny, 1995). Elucidation of allergic reactions has shown them to be caused more frequently by egg-white than egg yolk (Wüthrich, 1981; Anet *et al.*, 1985). Ovomucoid (OM), ovalbumin (OA), ovotransferrin (OTr) and lysozyme (LY) have all been identified as major allergens in egg-white protein (Langeland, 1982; Hoffman, 1983; Langeland & Harbitz, 1983; Holen & Elsayed, 1990). Over the past few years, most studies have focused on the allergenic structures of OA

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(Kahlert *et al.*, 1992; Elsayed, 1993; Honma *et al.*, 1996), which is the main protein in egg-white (54% of total protein).

Recently, OM has been reported to be the dominant allergen of egg-white in egg-allergic children (Bernhisel-Broadbent *et al.*, 1994). Several immunogenic studies of peptides derived by chemical or enzymatic fragmentation of OM have been carried out by different groups using specific mouse IgE or rabbit anti-OM sera (Kurisaki *et al.*, 1981, Matsuda *et al.*, 1985).

OM is a glycoprotein with a molecular weight (MW) of approximately 28 kDa and comprises about 11% of total egg-white proteins (Lin & Feeney, 1972). It consists of 186 amino acids building three tandem domains, each of which provide trypsin inhibitory activity (Kato *et al.*, 1987). The carbohydrate moieties of OM represent up to 25–30% of the whole MW. *N*-Acetylglucosamine (GlcNAc), mannose (Man), galactose (Gal) and *N*-acetylneuramic acid (NeuNAc) are the main saccharides in OM making up about 14–16, 6.5–8.5, 0.5–4 and 0.04–2.2% of the total molecular weight respectively (Beeley, 1971). The glycosylation sites are Asn¹⁰, Asn⁵³, Asn⁶⁹, Asn⁷⁵ and Asn¹⁷⁵. There are glycosylated and non-glycosylated isoforms of OM at Asn¹⁷⁵ (Kato *et al.*, 1987). The role of the carbohydrate moieties in the biological activity of OM has been a controversial subject of several investigations. For example, Gu *et al.* (1989) found no change in trypsin inhibitory and immunological activity of hen's egg OM after chemical deglycosylation, but suggested that the carbohydrate moieties contribute to the stability of OM against tryptic hydrolysis and heat denaturation. In contrast, Dabich *et al.* (1993) observed decreasing trypsin inhibitory activity of deglycosylated OM from turkey's egg. The specific interaction of carbohydrate epitopes in IgE binding has been described for different allergens. An *N*-glycan from honey bee venom phospholipase A₂ has been demonstrated to represent an IgE recognizing epitope (Weber *et al.*, 1987; Tretter *et al.*, 1993). Mucci *et al.* (1992) found no differences in specific IgE reactivity against native and deglycosylated *Parietaria judaica* pollen extracts. More recently, Petersen *et al.* (1995) reported that the carbohydrate moieties of the timothy grass pollen allergen *Phl p* I were recognized by IgE antibodies of one patient's serum. The influence of the carbohydrate moieties on the allergenicity of OM remains to be investigated.

The aim of the present study was to verify the contribution of the carbohydrate moieties of OM to allergen-antibody recognition at the B-cell epitope level. Deglycosylated OM (d-OM) was therefore prepared using a mild chemical deglycosylation method with trifluoromethanesulphonic acid (TFMS). IgE-binding properties of native OM and d-OM were compared by means of SDS-PAGE/immunoblot and enzyme-allergosorbent test (EAST) inhibition. The sera from a total of 33 egg-allergic patients were included.

MATERIALS AND METHODS

Human Sera

All patients' sera were RAST class ≥ 3 when tested for specific IgE against egg-white extract using CAP-RAST (Pharmacia, Uppsala, Sweden). Twenty-six patients were aged up to 10 years and seven were more than 20 years old. Pool serum was collected using equal aliquots of 10 sera with specific IgE binding against purified OM. A serum from a non-atopic individual with no history of food hypersensitivity (RAST class 0) was used as negative control.

Purification of Egg-white Proteins

OA (albumin from hen's egg-white), OM (trypsin inhibitor from hen's egg-white) and OTr (ovotransferrin = conalbumin from hen's egg-white) were purchased from Fluka (Buchs, Switzerland). LY from egg-white was obtained from Serva (Heidelberg, Germany). Commercial protein preparations were purified by size exclusion chromatography (SEC)

following ion exchange chromatography (IEC), performed with a high-performance liquid chromatography (HPLC) pump (L-6000) and UV detector (L-4000) from Merck (Darmstadt, Germany). Both methods were carried out at a flow rate of 1 ml min⁻¹ and a detection wavelength of 280 nm. IEC was carried out on a Resource Q column (1 ml; Pharmacia, Uppsala, Sweden) applying 10 mg of crude OA and OM per run. The eluent was 20 mM Tris-HCl (pH 8.0) with a salt gradient from 0 to 0.5 M-NaCl. The collected fractions were purified by SEC in 50 mM-Na₂HPO₄/0.15 M-NaCl/HCl (pH 7.0) on a Sephacryl S-100 HR column (C 26/100, Pharmacia). Eluted fractions were desalted and concentrated by ultrafiltration using Centriplus Concentrators (Amicon, Beverly, MA, USA) with a 10-kDa cut-off membrane. The resulting solutions (final volume of 300–500 µl in double distilled water) were stored until use at -20°C.

Deglycosylation of Ovomucoid

A GlycoFree Deglycosylation Kit (Oxford GlycoSystems, Abingdon, UK) was used to cleave the carbohydrate moieties from OM. Deglycosylation of 0.5 mg of purified, freeze-dried protein per reaction vial was carried out according to the manufacturer's instructions. Deglycosylation with anhydrous TFMS containing toluene was complete after 4 h at -20°C. After neutralizing and diluting steps, the reagents were removed by ultrafiltration using Centriplus Concentrators (Amicon, Beverly, MA, USA) with a 3-kDa cut-off membrane, resulting in a final volume of 200–400 µl in double distilled water. The solutions were stored at -20°C until use.

Spectrophotometric Analysis

Protein determinations of purified and deglycosylated proteins were done using the method of Lowry *et al.* (1951). The carbohydrate content was determined by phenol/sulphuric acid reaction according to Dubois *et al.* (1956) using a mixture of glucosamine (GlcN), Man and Gal (9/4/1, w/w/w) as the reference standard.

Matrix-assisted Laser Desorption/Ionization Time of Flight (MALDI-TOF) Mass Spectrometry

Mass spectra of OM and d-OM were obtained using the linear mode on a reflex time-of-flight TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) equipped with a 337-nm nitrogen laser. In general, 20–50 single-shot spectra were added to obtain a good signal-to-noise ratio. The mass spectrometer was calibrated with trypsinogen and carbonic anhydrase. Approximately 60 µg of lyophilized OM and d-OM were redissolved in 100 µl of saturated sinapic acid in 50% acetonitrile containing 0.1% trifluoroacetic acid. Prior to analysis, 10 µl of this solution were dried on the target.

Capillary Zone Electrophoresis

Egg-white protein separation was performed on a HP^{3D} capillary electrophoresis system (Hewlett-Packard, Wilmington, DE, USA). An underivatized fused-silica capillary (CS-Chromatographie Service, Langerwehe, Germany) of total length 64.0 cm, effective length 55.5 cm and inner diameter 100 µm was pre-conditioned before each run by flushing with 0.1 M-NaOH (1 min) and separation buffer (5 min). For the quantification of LY, OA and OTr, and the qualitative characterization of OM and d-OM, 20 mM-sodium phosphate (pH 3.5) containing 0.05% (w/v) polyethylene glycol 35000 (Merck) was used as a separation buffer. The OM contents were determined using 100 mM-formic acid (pH 2.2) as a separation buffer. Samples with protein concentrations from 0.001 to 10 mg ml⁻¹ were injected using pressure (50 mbar, 5 s). Separation was carried out by applying 30 kV at 25°C. Detection wavelengths were monitored at 195, 214 and 280 nm.

SDS-PAGE

SDS-PAGE was performed in slab gels (120, 220, 0.5 mm) with 5% (w/v) acrylamide stacking gel and 12% (w/v) acrylamide resolving gel according to the method of Lämmli (1970). Protein samples dissolved in Tris-HCl/SDS sample buffer (pH 6.8) containing 5% (w/v) β -mercaptoethanol were boiled for 5 min. Applying 2.5 μ g protein cm^{-1} , electrophoresis was performed at 200 V for 0.5 h to allow samples to invade the stacking gel, and at 600 V constant voltage for 2 h at 15°C on a Multiphor II horizontal slab electrophoresis unit (Pharmacia LKB) and MultiDrive XL power supply (Pharmacia LKB). Gels were silver stained according to the method of Morissey (1981).

Immunoblot

Proteins were electrotransferred from slab gels to nitrocellulose (NC membrane 0.2 μ m, Schleicher & Schuell, Dassel, Germany) at 0.8 mA cm^{-2} for 45 min using a NovaBlot semidry blotting apparatus (Pharmacia) according to the method of Kyhse-Andersen (1984) using a discontinuous buffer system as described by Vieths *et al.* (1992). Afterwards the membrane was dried for 30 min and cut into 5-mm strips.

Immunostaining of IgE was performed by the method of Vieths *et al.* (1992) modified slightly as described previously by Möller *et al.* (1997). In brief, dried membrane strips were blocked twice (15 min) in phosphate-buffered saline (PBS) containing 0.3% (v/v) Tween 20 and incubated overnight with 1 ml of each serum diluted 1:10 in PBS containing 0.3% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Tween 20 at room temperature.

Strips were then incubated consecutively with rabbit anti-human IgE (1:4000, DAKO, Copenhagen, Denmark), biotinylated goat anti-rabbit IgG (1:6000, DAKO) for 1 h each and with streptavidin-horseradish peroxidase (1:20 000, Medac, Hamburg, Germany) for 20 min. 3,3',5,5'-Tetramethylbenzidine and diocetyl sodiumsulphosuccinate were used as substrates for staining.

From each gel, one strip including separated proteins and an mw marker (LMW, Pharmacia) was stained with Indian ink according to Hancock and Tsang (1983).

EAST Inhibition

For protein adsorption, MaxiSorp-tubes (5.0 ml, Nunc, Roskilde, Denmark) were incubated overnight at 4°C with 100 μ l of a solution of purified OM in 50 mM-carbonate/bicarbonate buffer (pH 9.6) at a concentration of 10 μ g ml^{-1} . Non-specific binding sites were blocked with 200 μ l of PBS containing 1% (w/v) BSA for 1 h at room temperature.

For inhibition experiments, an equal volume of the pool serum (diluted 1:5 in PBS containing 1% (w/v) BSA and 0.5% (v/v) Tween 20) was added to inhibitor proteins diluted in the same buffer and pre-incubated for 30 min. Purified OM and d-OM were used as inhibitors in concentrations from 0.1 ng ml^{-1} to 1 mg ml^{-1} . Non-specific inhibition was checked with casein. After washing the tubes with PBS containing 0.05% (v/v) Tween 20 the pre-incubated solutions (100 μ l) were added, incubated for 3 h at room temperature and washed again. Finally, specific IgE was measured using an EAST test kit (Allergopharma, Reinbek, Germany) following the manufacturer's instructions. Absorbance was measured at 405 nm.

RESULTS

Purification of Egg-white Proteins and Characterization of Human Sera

Capillary zone electrophoresis (CZE) of commercial protein preparations showed impurities of 3.5% LY, 10% OM and 11% OTr in crude OA and 1.8% LY in crude OM. The corresponding SDS-PAGE bands were detected at 14.5 kDa (LY), 30–43 kDa as a single broad band (OM), 43 kDa (OA) and 77 kDa (OTr). In order to avoid overlapping of OA

TABLE 1. Characterization of patients' sera with IgE binding to OA and/or OM

Serum no.	RAST ^a	Age ^b	OA ^c	OM ^c
1	3	A	+	+
2	5	Ch	+	—
3	5	Ch	+	—
4	4	Ch	+	—
5	3	Ch	+	+
6	3	Ch	+	—
7	6	A	+	—
8	3	A	+	+
9	3	A	—	+
10	3	Ch	—	+
11	4	Ch	—	+
12	4	Ch	—	+
13	5	Ch	—	+
14	3	Ch	—	+
15	5	Ch	+	+
16	5	Ch	—	+
17	3	Ch	+	+

^aSpecific IgE against egg-white in six RAST classes.

^bAge of patients: children (Ch) < 10 years, adults (A) > 20 years.

^cIgE binding in SDS-PAGE/immunoblot: positive (+), negative (—).

and OM bands in SDS-PAGE/immunoblot and cross-reactivity in EAST inhibition, purification of OA and OM was necessary. After IEC/SEC purification, desalting and concentrating, the purity was checked again by CZE. It was demonstrated that purified OA and OM contained no detectable impurities of other proteins. Detection limits were below 0.1% for LY, OA and OTr and below 0.5% for OM. Furthermore, no IgE binding was observed when testing purified OA with a serum specific only for OM and vice versa in SDS-PAGE/immunoblot. Sera from 33 patients with specific IgE against egg-white were tested against purified OA and OM. A total of 17 sera (51.5%) gave positive reactions as shown in Table 1. IgE binding to OA was observed in 10 sera (30.3%) and to OM in 12 sera (36.4%). In five sera (15.2%), IgE binding to both allergens was demonstrated, whereas the IgE of five sera (15.2%) exclusively bound to OA and seven sera (21.2%) showed exclusive binding to OM. Sixteen sera (48.5%) did not bind to OA or OM and were therefore excluded from further experiments.

Deglycosylation of Ovomucoid

Chemical deglycosylation of purified OM was monitored electrophoretically by SDS-PAGE and CZE. In SDS-PAGE, purified OM was detected at 30–43 kDa while d-OM was detected at 21–28 kDa, both as single diffuse bands. Purified OM migrated in CZE with a broad shape showing three zones and two additional shoulders at migration times of 14–22 min (Figure 1(a)). For d-OM, shorter migration times of 8–13 min were observed. Although the peak was smaller for d-OM, it still showed almost the same pattern of five isoforms (Figure 1(b)).

Exact loss of MW after deglycosylation was measured by MALDI-TOF mass spectrometry. The average MW of OM was 28 kDa. The distribution of unresolved heterogeneous species was in the range 25 000–31 000 m/z and 12 500–15 000 m/z for single- and double-charged molecule ions respectively (Figure 2(a)). Mass spectra of d-OM showed five distinct isoforms with MWs of 20.699, 20.895, 21.096, 21.299 and 21.501 kDa. Double-charged molecule ions were detected at 10 500–10 800 m/z (Figure 2(b)).

The carbohydrate content was measured spectrophotometrically by the phenol/sulphuric acid method. Purified OM contained 31.1% carbohydrates referring to a carbohydrate

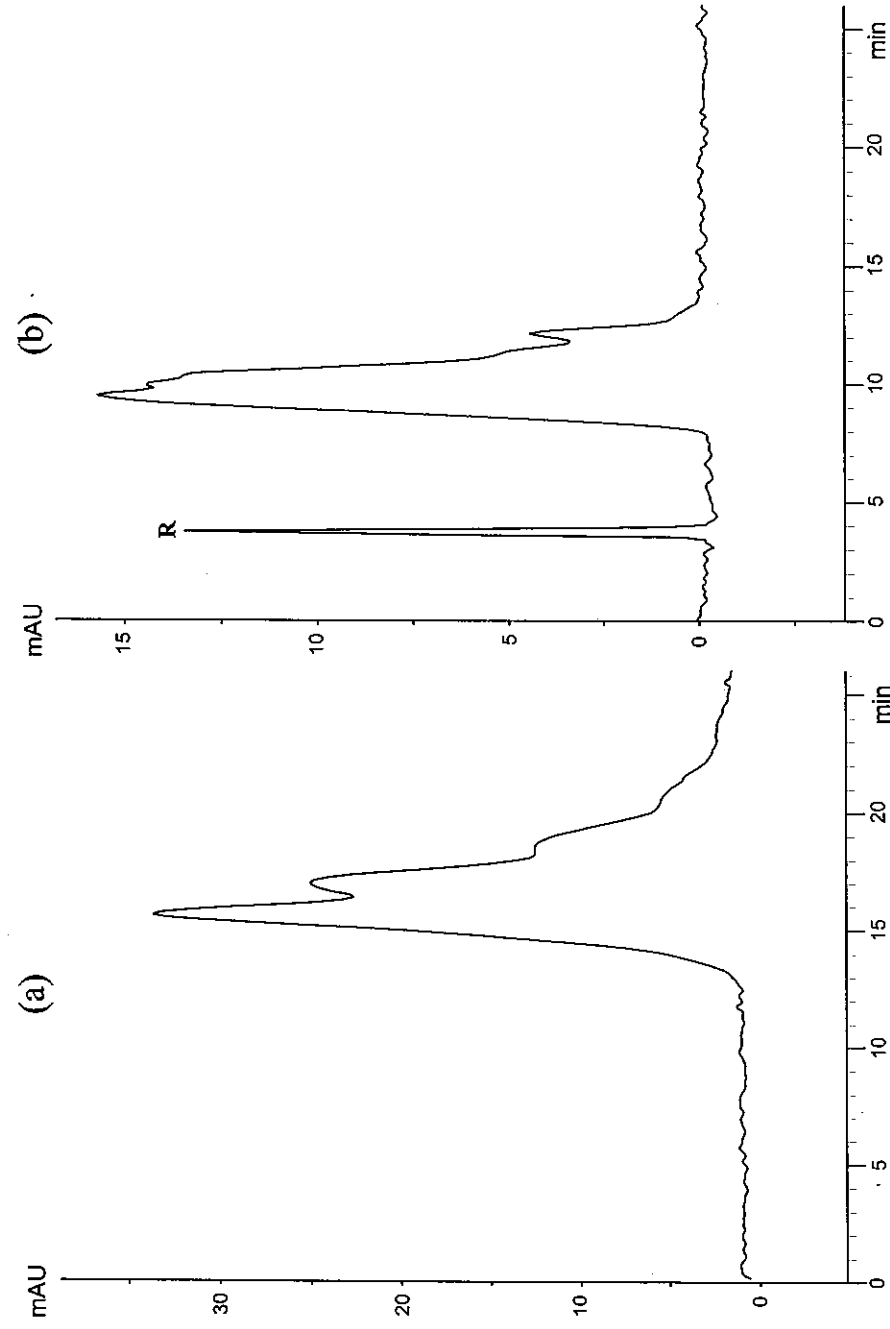


FIG. 1. Comparison of the migration times and resolution of different isoforms from OM and d-OM using CZE with phosphate buffer (pH 3.5) and a detection wavelength at 214 nm. (a) Electropherogram of native OM. (b) Electropherogram of d-OM containing reagent peak R (mAU = milli absorption units).

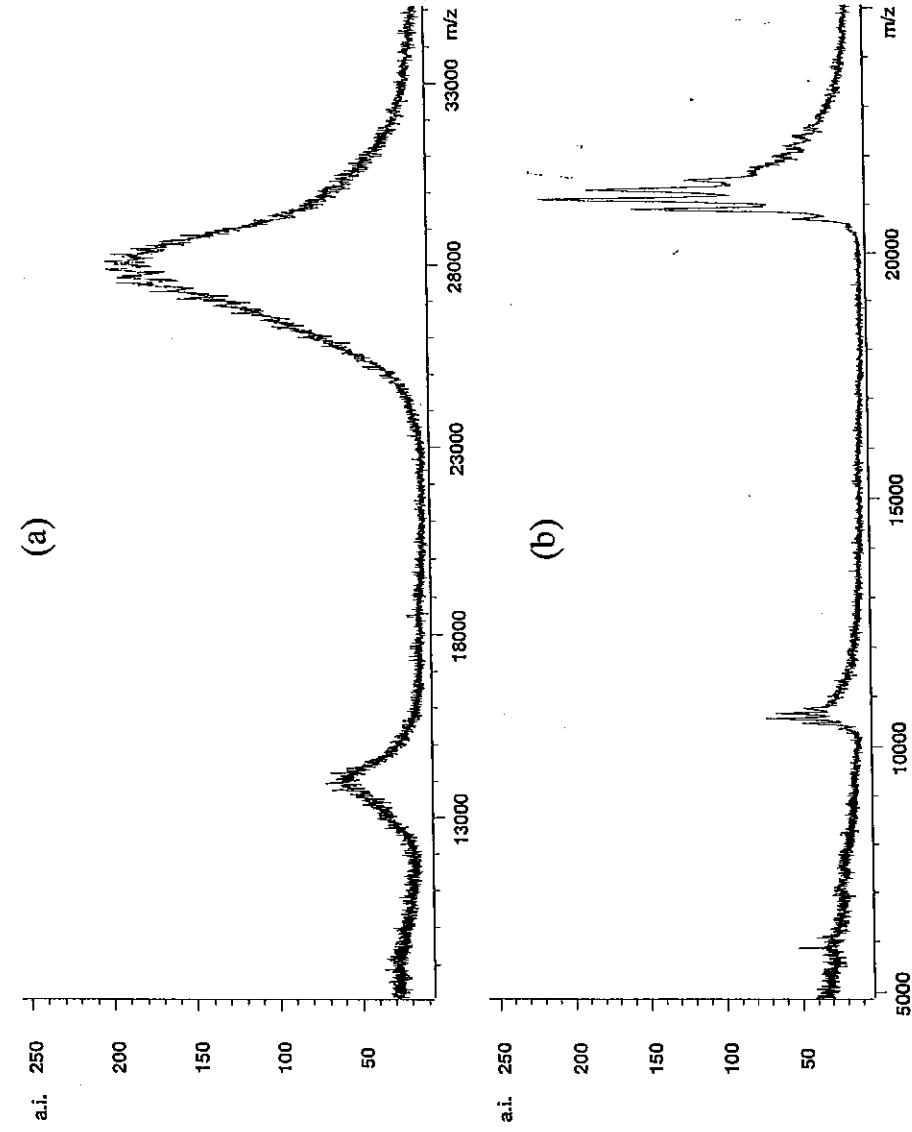


FIG. 2. MALDI-TOF mass spectrometry of (a) native OM and (b) d-OM showing the single-charged molecule ions at 25 000–31 000 m/z (OM) and 20 699–21 501 m/z (d-OM) and the double-charged molecule ions at approximately 12 500 to 15 000 m/z (OM) and 10 500–10 800 m/z (d-OM) (a.i. = arbitrary intensity).

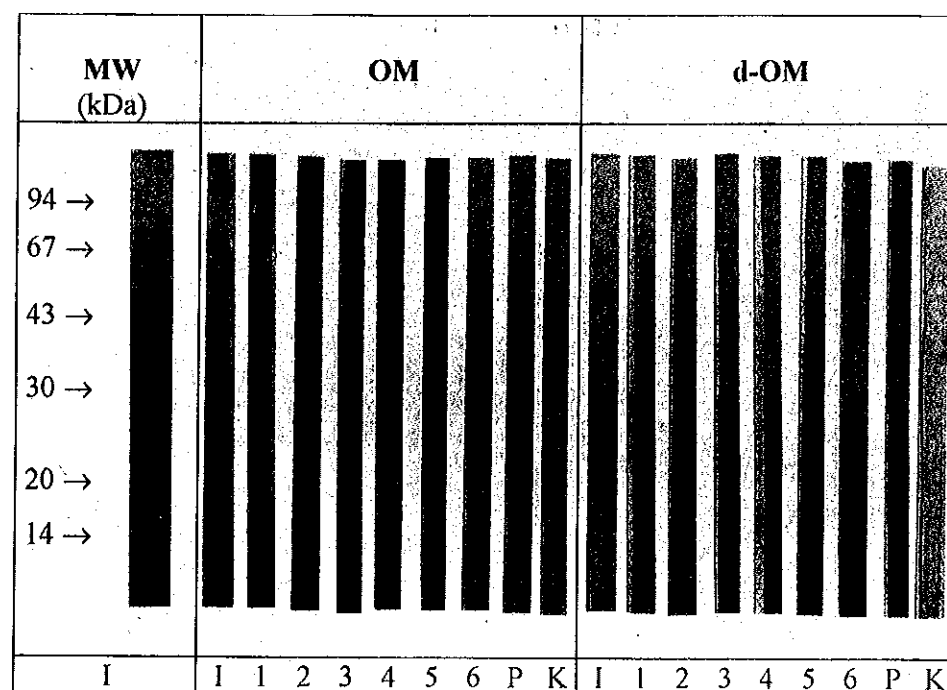


FIG. 3. SDS-PAGE/immunoblot of OM and d-OM. Immunostaining: 1-6 = patients' sera, P = pool serum, K = negative control; unspecific staining: I = Indian ink, MW = molecular weight marker.

mixture composed of GlcN, Man and Gal. NeuNAc as a minor component was not included in the standard mixture. The carbohydrate content of d-OM was about 0.5%. Therefore the carbohydrate content of OM was reduced about 98.4%.

SDS-PAGE/Immunoblot

Purified OM and d-OM were tested using SDS-PAGE/immunoblot. Owing to the small amount of patients' sera, not all the 12 sera showing positive IgE binding against OM could be tested. Ten sera were included in the pool serum and six sera were able to be used for single investigations. All the sera tested (two of them detecting OM exclusively and four detecting OM and OA) and the pool serum showed strong IgE binding to OM and d-OM (Figure 3). Weak IgE binding to bands at approximately twice the MW of OM and d-OM was observed.

EAST Inhibition

The inhibitory activity of OM and d-OM against OM adsorbed to the surface of a test-tube was measured by EAST inhibition. Since all the sera tested showed the same IgE binding using SDS-PAGE/immunoblot the diluted pool serum was used. OM gave 50% inhibition of IgE binding at a concentration of 67 ng ml^{-1} , while d-OM reached 50% inhibition at 57 ng ml^{-1} (Figure 4).

DISCUSSION

The purification steps employed resulted in highly purified protein preparations, which were suitable for immunological investigations. CZE was a rapid and sensitive method for monitoring the purification of egg-white proteins. Ebbelhøj *et al.* (1995) found a detection

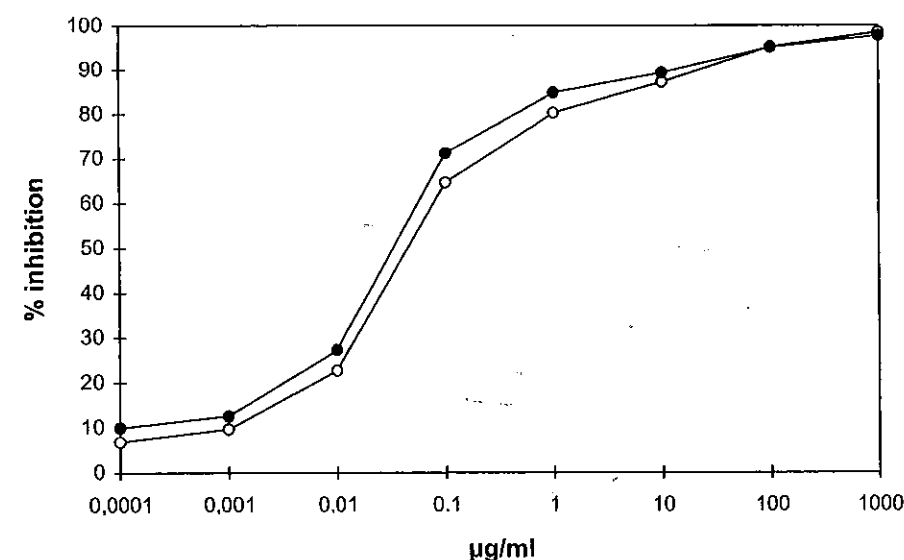


FIG. 4. EAST inhibition of IgE binding to OM with OM (-○-) and d-OM (-●-) as inhibitors.

limit of 0.1% when analyzing contaminating proteins in purified egg-white proteins by silver staining and crossed immunoelectrophoresis. These results are in the same range as those obtained by CZE in this investigation. Furthermore, CZE requires no sample reduction, gel preparation, staining procedures or specific antibodies and therefore the analysis time for a few samples is much shorter. SDS-PAGE/immunoblot showed that the frequency of IgE binding from patients' sera to OA and OM was in agreement with the results obtained by other authors, for example Aabin *et al.* (1996), who reported that 32-38% of 34 sera from egg-allergic patients contained specific IgE against OA and OM respectively. It was confirmed that OA and OM are frequent allergens in egg-white.

The applied TFMS deglycosylation is reported to hydrolyze glycosidic linkages without affecting peptide bonds (Edge *et al.*, 1981; Dabich *et al.*, 1993). Complete deglycosylation of OM was assumed by a decrease in MW as determined by SDS-PAGE and MALDI-TOF mass spectrometry. Differing results for intact OM using SDS-PAGE (30-43 kDa) and MALDI-TOF (28 kDa) were observed. Both results are in agreement with data reported for SDS-PAGE and physicochemical methods respectively (Burley & Vedehra, 1989; Ebbelhøj *et al.*, 1995). Overestimation of the MW for OM and d-OM from turkeys' eggs was observed in SDS-PAGE by Dabich *et al.* (1993). The lower electrophoretic mobility of OM may be due to a lower SDS/protein ratio since carbohydrate moieties do not bind SDS. The broad shapes of OM and d-OM bands in SDS-PAGE could be caused by incomplete reduction of the nine disulphide bridges hindering formation of unique ellipsoid SDS-protein complexes, although acetylation with iodoacetamide did not improve the shape of the bands (data not shown). To measure the change in MW exactly, MALDI-TOF mass spectrometry was the preferred method. The decrease in MW was about 23-26%, which is in agreement with reported carbohydrate contents of 22-29% in different OM isoforms (Beeley, 1971).

The 98.4% carbohydrate removal from OM as measured spectrophotometrically was even higher than that described by Gu *et al.* (1989) and similar to that reported by Dabich *et al.* (1993), who removed about 75 and 94% by TFMS treatment respectively. Since hydrolysis with TFMS does not release GlcNAc residues attached to Asn (Edge *et al.*, 1981; Tams & Welinder, 1995) the five isoforms of d-OM measured by MALDI-TOF may be due to uncleaved GlcNAc. The observed differences in MW between the two deglycosylated

isoforms (21.096 and 21.299 kDa) and pure OM peptide chain (calculated at 20.080 kDa) correspond to five and six GlcNAc residues respectively. Both MALDI-TOF and CZE analysis revealed five isoforms of d-OM. Whether the isoforms separated by these methods correspond to each other, could not be concluded due to the different separation mechanisms used. Whereas for native OM five isoforms could be separated using CZE, no distinguished isoforms were observed by MALDI-TOF. The occurrence of these isoforms may correspond to the five *N*-glycosidic linkages in OM. Although CZE provides a resolution of the five isoforms of native OM, no further conclusions could be drawn about the structural composition. In contrast the average MW of native OM could be determined by MALDI-TOF, but no isoforms could be differentiated. However, OM and d-OM showed different electrophoretic mobilities as determined by CZE, thus CZE was a less time-consuming alternative to SDS-PAGE for monitoring deglycosylation of OM.

d-OM was detected by all tested sera containing specific IgE against OM in SDS-PAGE/immunoblot at almost the same intensities as OM itself. Therefore, each serum showed strong IgE binding to epitopes on the protein backbone. The weakly detected bands at twice the MW indicating the formation of dimers were not detected by silver staining. Therefore, these dimers were at a very low concentration, probably below 0.1% of the monomers as estimated by the detection limit of silver staining.

d-OM reached 50% inhibition of IgE binding in EAST at very similar, but slightly lower, concentrations as OM. The two inhibition curves were almost parallel, indicating that OM and d-OM share the same epitopes. The increased inhibition capability could be due to a better steric access in epitopes after the removal of carbohydrate residues. Deglycosylation caused no loss in IgE binding epitopes on OM. Therefore, carbohydrate structures do not participate in IgE binding. After oxidation of OM, in order to destroy carbohydrate residues, Cooke *et al.* (1996) concluded that the carbohydrate moieties of OM participate in IgG binding. Furthermore they indicated that there was no significant role for the carbohydrate moieties of OM in IgE binding, but no data were presented. Our results obtained using a mild and efficient deglycosylation method proved that OM allergenicity is independent of the carbohydrate moieties.

CONCLUSIONS

It has been demonstrated that the carbohydrate moieties of OM have no impact on IgE recognition at the B-cell epitope level in egg-allergic patients. Future work should be focused on investigations into the allergenic structures of the peptide chain. Epitope mapping by fragmentation of OM and synthetic or recombinant peptides should be undertaken to locate amino acid sequences of IgE-binding sites.

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Immunochemical Identification of AFLR, a Regulatory Protein, Involved in Aflatoxin Biosynthesis

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Polyclonal antibodies against AFLR, the aflR gene product of *Aspergillus flavus* and *A. parasiticus*, were generated by immunizing a rabbit with the *Escherichia coli*-expressed recombinant AFLR protein of *A. flavus*. Immunoblot analysis revealed that the antibodies not only reacted with the recombinant AFLR protein of *A. flavus* or *A. parasiticus* but also with native 47-kDa AFLR in *A. flavus* and *A. parasiticus*. Immunoblot analysis revealed that accumulation of the 47-kDa AFLR in cultures of *A. flavus* and *A. parasiticus* correlated well with the production of aflatoxin under various culture conditions that regulate aflatoxin formation. Neither AFLR nor aflatoxin was found when *A. parasiticus* NRRL 2999 was grown in peptone mineral salts (PMS) medium; however, both were detected after the culture was transferred to glucose mineral salts (GMS) medium. The AFLR protein was absent in the non-aflatoxigenic *Penicillium* and *Fusarium* species grown in GMS medium. The data indicate that the antibodies obtained in the present studies are specific for AFLR and could be used in various studies to monitor the role of AFLR in regulating aflatoxin biosynthesis.

Keywords: Aflatoxin, AFLR, antibodies, regulation, *A. flavus*, *A. parasiticus*

INTRODUCTION

Aflatoxins are a group of toxic secondary metabolites produced by certain strains of *Aspergillus flavus* and *A. parasiticus* which contaminate many agricultural commodities such as corn, peanut, tree nuts and cottonseed (CAST, 1989; Jelinek *et al.*, 1989). Aflatoxin B₁ (AFB₁), the most toxic compound in this group, is one of the most potent naturally

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