

ORIGINAL PAPER

Genetically glycosylated ovomucoid third domain can modulate Immunoglobulin E antibody production and cytokine response in BALB/c mice

P. Rupa*, S. Nakamura[†] and Y. Mine*

*Department of Food Science, University of Guelph, Guelph, ON, Canada N1G2W1 and [†]Food Biotechnology Laboratory, Graduate School of Agricultural Science, Shinshu University, Nagano, Japan

Clinical and Experimental Allergy

Summary

Background Food allergies are on the rise and it is estimated that in North America, 8% of the children and 4% of the adults have food allergies. Food allergies tend to occur more often in children than in adults due to their immature digestive and immune systems. Hen's egg is among the most common cause of food-induced allergic reactions in North America.

Objective The present study was undertaken to investigate the role of N-glycans of the third domain of ovomucoid in IgE binding and modulation of allergen-specific immune response in BALB/c mice.

Methods The cDNA encoding the third domain of ovomucoid was inserted into the yeast genome and expressed in *Pichia pastoris* X-33 cells, under the control of the glyceraldehyde-3-phosphate (GAP) dehydrogenase promoter for constitutive expression to obtain a post-translationally modified and functionally active ovomucoid third domain. Upon expression, the protein was secreted into the extracellular medium and was purified by size exclusion chromatography. The recombinant protein was produced at 10 mg/L of the culture supernatant. BALB/c mice were sensitized with the recombinant and native forms of glycosylated ovomucoid third domain antigen. The allergic response of the native and the recombinant glycosylated forms of ovomucoid third domain antigens were compared using antibody and cytokine measurements.

Results ELISA tests indicated a significant decrease in specific IgE antibodies to the recombinant N-linked glycosylated form (P-Gly), when compared with the native glycosylated form (DIII⁺) using mice sera. Immunization with P-Gly induced the production of IFN- γ [T-helper type 1 (Th1) response] and lowered the production of IL-4 (Th2 response), and a skewed balance towards the Th1 cytokine demonstrated that P-Gly has a modulating ability on Th1/Th2 balance to down-regulate Th2 response. Furthermore, N-linked glycan (N28) in the third domain of ovomucoid was shown to be associated with suppression of the allergic response.

Conclusion Therefore, we can conclude that P-Gly facilitates and contributes to the discovery of new molecular target for the development of a safe and specific therapeutic vaccine for the treatment of egg allergy, and oligosaccharides do seem to play a major role in the suppression of IgE-binding activity.

Keywords food allergy, IgE, N-linked glycosylation, ovomucoid, *Pichia pastoris*, Th1/Th2

Submitted 10 October 2006; revised 4 March 2007; accepted 12 March 2007

Correspondence:

Dr Yoshinori Mine, Department of Food Science, University of Guelph, Guelph, ON, Canada N1G2W1.

E-mail: ymine@uoguelph.ca

Background

Ovomucoid is a glycoprotein in the hen's egg with a carbohydrate content of about 22–29% of the whole molecule, present as oligosaccharides, each joined to the

polypeptide chain by an asparaginyl (Asn-N) residue [1]. Glycosylation, the attachment of carbohydrate chains to proteins, is a crucial part of biology, and half of all proteins encoded by the genome get sugars attached to them at some point or the other once they are made. There

are glycosylated and non-glycosylated isoforms of ovomucoid at the Asn-175 position. The molecular weight of the oligosaccharides of ovomucoid is estimated to be between 1467 and 2441 Da [2]. These sugar chains are mostly larger than a glucose dodecamer classified into three groups: N-1, N-2 and N-3b. N-1 is a mixture of two isomeric digalactosyl derivatives of N-3b, N-2 is a mixture of three isomeric monogalactosyl derivatives of N-3b and N-3b is the structure found in all pentaantennary sugar chains. The main saccharides of ovomucoid are *N*-acetylglucosamine (GlcNAc), mannose (Man), galactose (Gal) and *N*-acetylneuramic acid (NeuNAc) with about 14–16%, 6.5–8.5%, 0.5–4% and 0.04–2.2% of the total molecule, respectively [3]. Glycosylation sites are Asn-10, Asn-53, Asn-69, Asn-75, Asn-158 and Asn-175. Covalent multi-antennary structures of glycans are present in ovomucoid. It was shown that the carbohydrate chain attached to the third domain of ovomucoid played an important role in its binding activity against human IgE antibodies [4]. About 30% of the serum antibody to ovomucoid reacted with the carbohydrate-containing domain, whereas little or no antibody reactivity was detected against the carbohydrate-free domain. This suggests that the carbohydrate chain attached to the third domain played an important role in antigenic determinants of ovomucoid. In contrast, it was shown that carbohydrates did not participate in the allergenicity of ovomucoid and after removal of about 98.4% of carbohydrates from ovomucoid, there were no differences in IgE binding of ovomucoid and deglycosylated ovomucoid by SDS-PAGE/immunoblotting and inhibition experiments [5]; rather, the carbohydrate moieties in DIII had inhibiting effects on its IgG- and IgE-binding activities [6]. IgE responses to N-linked glycan moieties of glycoproteins are frequent in patients, but their clinical relevance still remains controversial [7].

Recently, studies suggested that glycan moieties (N-glycans) did not seem to constitute a relevant IgE epitope in the major pollen allergen: the Pla I 1 [8]. On the contrary, carbohydrate moieties did seem to play a role in the IgE and T cell response of Japanese cedar pollen allergen [9]. Thus, the role of carbohydrate moieties in the allergenic activity of allergens is still a controversial issue. Post-translational modifications are known to have an impact on the IgE-binding activity [10].

The *Pichia pastoris* system has been used to study site-specific glycosylations and enhancement of the molecular stability of recombinant proteins [11]. The number of mannose residues ('high-mannosylation' – glycosylation pattern) added to *P. pastoris* recombinant glycoproteins are mainly Man₈GlcNAc₂ and Man₉GlcNAc₂. The native ovomucoid third domain contains two N-linked motifs at amino acid position 28 (corresponding to position 158 in intact ovomucoid) and 45 (corresponding to 175 in intact ovomucoid). The N-linked glycosylation site at position 28 is not found in a naturally glycosylated state, whereas

position 45 is naturally glycosylated. In order to determine the impact that each glycan motif has on the structure and function of the glycoprotein, the third domain of ovomucoid was expressed in the *P. pastoris* eukaryotic system in a genetically glycosylated form, and in this case, glycosylation is shown to occur at random at both the N-linked motifs (28 and 45). The advantage of using this system is that yeast does not encounter the endotoxin problem that is associated with bacteria nor the viral contamination problem of proteins produced in animal cell culture. The genetic structure of the yeast allows it to produce high levels of protein, without adverse effects to itself or to the protein. *Pichia pastoris* have been developed as an effective production system for recombinant proteins, offering economy, ease of manipulation, the ability to perform complex post-translational modifications and exhibit high expression levels [12]. The proteins produced in *P. pastoris* are typically folded correctly and secreted into the medium and provide an excellent alternative to *Escherichia coli* expression system.

In order to evaluate the effect of glycosylation on the IgE-binding reactivity of the allergen, we have focused on studying the immunological response to the third domain of ovomucoid after post-translational modification, by cloning the ovomucoid third domain molecule in the *P. pastoris* yeast system. Ovomucoid has nine cysteine residues and since the yeast *P. pastoris* has been reported to be a powerful system for expression of proteins containing cysteine residues and disulphide bonds. It also allows secretion of soluble proteins with very high yields after post-translational modification [13] and thus yeast is considered as an ideal system. A number of proteins have been successfully cloned and expressed in a yeast system [14–16]. The ovomucoid third domain (DIII) in this study was expressed under the control of the *Pichia* glyceraldehyde-3-phosphate dehydrogenase (pGAP) promoter. The pGAP promoter was isolated by Waterham et al. [17] and has been successfully used for the constitutive expression of several heterologous proteins. As per the Asn–Xxx–Thr consensus sequence for N-linked glycosylation, the secreted protein, glycosylated at positions 28 and 45 (N-linked glycosylation sites of the third domain of ovomucoid), had an apparent molecular weight of 6 kDa. The immunological response of the allergen was characterized using a murine model system (BALB/c) and it was found that the glycosylated allergen suppressed IgE response to about 50%. There was an inclination and immune-deviation towards a T-helper type 1 (Th1)-biased response. Our results suggest that high yields of glycosylated ovomucoid third domain can be obtained and based on analysis of the data, the genetically glycosylated ovomucoid is shown to be involved in inhibition of allergic response and can be a potential target for immunotherapy to treat egg-white allergy.

Materials and methods

Animals and reagents

Female BALB/c mice were purchased from Charles River Laboratories (Mississauga, ON, Canada), and maintained in a pathogen-free environment at the Central Animal Facility, University of Guelph. The mice were maintained under an egg-free diet (Teklad Global 14% protein and 3.5% fat) and a 12/12 h lighting cycle for the duration of the experiment. Food and water were available *ad libitum*. *Pichia pastoris* X-33 yeast cells were used as hosts for plasmid pGAPZ α -A expression vector. Both pGAPZ α -A and X-33 were obtained from Invitrogen (San Diego, CA, USA). All antibodies were purchased from BD Biosciences (San Diego, CA, USA).

Construction of ovomucoid in the yeast expression system

The cDNA encoding the third domain region of ovomucoid, containing the amino acid region of 131–186 of the mature protein, was amplified by polymerase chain reaction (PCR) using the ovomucoid-specific cDNA as a template; two primers: (i) a sense primer 5'-GTATCTCTC GAGAAAAGACTTGCTGCTGTGAGTGTG-3' and (ii) an antisense primer 5'-TGGGGTTCTAGATTAGCATTTTCCAA AATGGC-3', were used to amplify the ovomucoid third domain fragment. The sense primer was designed to contain an *Xho*I restriction site (underlined), a sequence that allows the fusion of the encoding region in-frame with the sequence of the pGAPZ α -A plasmid, (Invitrogen) corresponding to the amino acid region of 1–6 of the mature ovomucoid protein (LAAVSV). The antisense primer was designed to contain an *Xba*I restriction site (underlined), a stop codon and has a sequence corresponding to the 181–186 amino acid region of the ovomucoid carboxy terminal end (CKGFHS). The PCR-amplified fragment was further digested with *Xho*I and *Xba*I restriction enzymes and gel purified and cloned into the *Xho*I–*Xba*I digested pGAPZ α -A plasmid vector (a shuttle vector for *E. coli* and *P. pastoris* system) and transformed into *E. coli* Top10 F' cells to obtain the construction of the pGAPZ α -A/ovomucoid third domain fragment (P-Gly). The schematic representation of the ovomucoid third domain construct cloned in pGAPZ α -A is shown in Fig. 1. The *E. coli* transformants were selected on plates containing the antibiotic, ZeocinTM (Invitrogen), at a concentration of 25 μ g/mL. Verification of insertion of the product in correct translational reading frame was confirmed by DNA sequencing, before the introduction of the chimeric plasmid into *P. pastoris* host cells. The recombinant plasmid containing the ovomucoid gene was linearized with *Rca*I restriction enzyme and the product was used to electroporate competent X-33 yeast cells using a Gene Pulser (Model No. 1652098, Bio-Rad Laboratories, Hercules, CA, USA) as recommended by the manufacturer (Invitrogen).

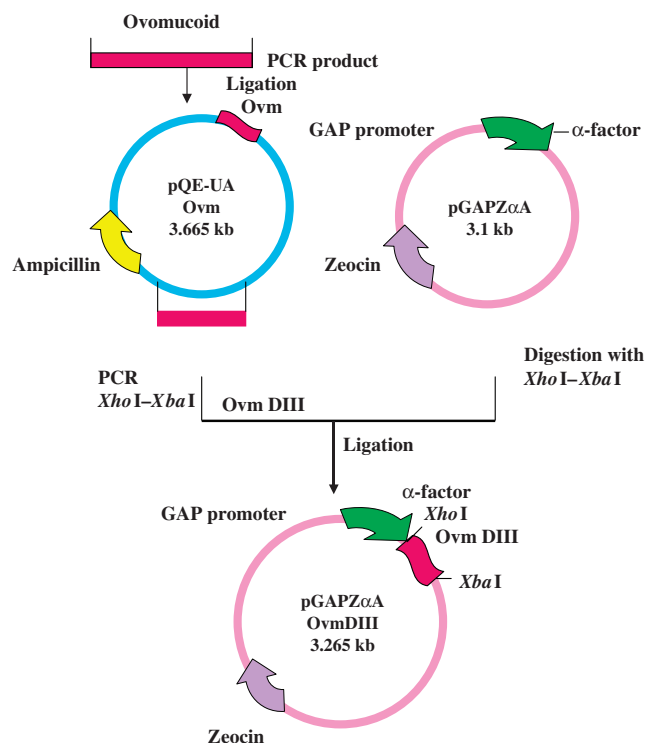


Fig. 1. Schematic representation of the cloning strategy used for the construction of glycosylated recombinant ovomucoid third domain (P-Gly).

Expression and purification of the recombinant ovomucoid in *Pichia pastoris*

YPDS (1% yeast extract, 2% peptone, 2% glucose and 1 M sorbitol) plates containing 2% sorbitol and zeocin (100 μ g/mL) were used for the selection of *P. pastoris* transformants. Single colonies were picked and inoculated into 10 mL of yeast extract/peptone/dextrose-containing medium and grown at 30 °C in a shaking incubator (250 r.p.m.). The following day, 200 μ L of the overnight culture was used to inoculate 100 mL of medium in a 250-mL baffled flask, and the culture was grown for 3 days. Samples were obtained every day to determine optimal expression levels. Cells were subjected to centrifugation (7000 g for 15 min). The extracellular medium obtained after centrifugation of the yeast cells was collected and the recombinant protein extraction was performed. The initial concentration of the supernatant using 100% PEG 8000 (Fischer Scientific, Pittsburgh, PA, USA) was carried out for 5–6 h and the sample was dialysed with 20 mM Tris buffer (pH 8.0) (3500 cut-off membrane; Fischer Scientific). Size exclusion chromatography on a Superdex HR-75 column (100 mL bed volume) was used to fractionate the sample. Protein concentrations were determined by DC assay method according to the Bio-Rad-Microassay procedure (Bio-Rad Laboratories). A standard curve was produced with known concentrations of bovine serum albumin (BSA) (Fischer Scientific). Eluates were analysed

by SDS-PAGE using carbohydrate-specific Schiff's (Sigma, Oakville, ON, Canada) staining. The native ovomucoid third domain antigen containing the carbohydrate chain (DIII⁺) was purified after hydrolysis with *Staphylococcus aureus* V₈ (Spase V₈) as reported earlier [1]. Domain III ovomucoid without the carbohydrate chain (DIII⁻) was prepared as described earlier [6].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis and Western blot analysis for detection of the ovomucoid glycoprotein

Periodic Acid-Schiff (PAS) stain was used to detect the bands on a 10% SDS-PAGE gel. This stain specifically identifies the carbohydrate-containing proteins that have been subjected to SDS-PAGE. Briefly, gel was soaked in acetic acid, periodic acid and Schiff reagent (Sigma) (Fuchsin sulphurous acid). Staining of sugar moieties of glycoproteins yields magenta bands (reddish pink) with a colourless background. Western blotting was performed according to the method of Burnette [18] with slight modifications. Glycoproteins were transferred onto a nitrocellulose membrane and bands were visualized, based on a detection protocol developed by Kondo et al. [19]. The transfer of proteins onto the nitrocellulose membrane was performed using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories) as directed by the manufacturer. Briefly, proteins were separated by SDS-PAGE gel and transferred electrophoretically onto a nitrocellulose membrane. After electro blotting, the nitrocellulose membrane was carefully removed and the glycoproteins on the nitrocellulose membrane were first oxidized by sodium periodate; biotin hydrazide in 0.1 N NaOH was then coupled to the aldehyde groups generated in the sugar moiety of the glycoproteins. The membrane was washed twice using sodium hydroxide, and avidin-horseradish peroxidase (1:2000) was then conjugated to the remaining biotin and bands were visualized by BCIP/NBT substrate solution (5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride) in 0.1 M Tris-HCl, pH 9.5, containing 0.1 M NaCl and 0.05 M MgCl₂ according to the manufacturer's instructions (Roche Diagnostics Corporation, Indianapolis, IN, USA).

Mice immunization

Seven-week-old female BALB/c mice were purchased from Charles River Laboratories and maintained in groups of four per cage at the Central Animal Facility, University of Guelph, in accordance with the standard guidelines from the Canadian Council on Animal Care ('Guide to the Care and Use of Experimental Animals'). The mice were maintained under an egg-free diet (Teklad Global 14% protein and 3.5% fat) and a 12/12 h lighting cycle for the

duration of the experiment. Food and water were available *ad libitum*.

Three sets of mice were used. The first set of mice ($n = 4$ /group) were injected subcutaneously with 50 µg of purified recombinant glycosylated ovomucoid (P-Gly) produced in *P. pastoris*, emulsified with an equal volume of Complete Freund's adjuvant (CFA) (Sigma). The second set of mice were injected with 50 µg of native glycosylated ovomucoid third domain antigen (DIII), and the third set of control mice were injected with 50 µL of phosphate-buffered saline (PBS) in Freund's adjuvant. Booster injections with incomplete Freund's adjuvant (IFA) (Sigma) were given on days 28 and 42. Two weeks after the last immunization, mice were humanely euthanized by CO₂ gas and blood samples were taken from the heart, serum was prepared and stored at -80 °C until further use. Spleens were removed individually and collected aseptically in ice-cold RPMI 1640 media (Gibco, Grand Island, NY, USA) and used for culturing splenocytes.

Detection of total and specific Immunoglobulin E

IgE antibody binding to purified ovomucoid antigen expressed in *Pichia* (P-Gly) and to native ovomucoid (DIII⁺) was measured by ELISA. Serum antibodies to recombinant glycosylated ovomucoid and native glycosylated ovomucoid were collected at the end-point from immunized mice. Briefly, flat-bottom 96-well ELISA plates (Corning Costar Corp., Cambridge, MA, USA) were coated with 100 µL of anti-mouse IgE monoclonal antibody (BD Biosciences) (1 µg/mL) diluted in 50 mM sodium bicarbonate buffer (pH 8.5) and incubated at 4 °C overnight. Plates were washed three times with 200 µL of 0.05% Tween 20 in PBS and then blocked with 200 µL of 2% BSA in PBS for 2 h at room temperature. The plates were washed three times with 200 µL of 0.05% Tween 20 in PBS, sera from each group of mice were diluted (1:20) in 1% BSA and 0.05% Tween 20 in PBS and 100 µL was added to the wells in duplicate and incubated for 2 h at room temperature. A duplicate dilution series of a standard mouse IgE antiserum (Serotec, Raleigh, NC, USA) was added to the plate. After extensive washing (four times) with 0.1% Tween 20 in PBS, plates were incubated with 100 µL of monoclonal anti-mouse IgE conjugated to biotin (Caltag, Burlingame, CA, USA) diluted at 1:1000 (final concentration of 0.4 µg/mL) in 1% BSA and 0.05% Tween 20 in PBS for 2 h at room temperature. The plate was further washed thrice with PBS containing 0.05% (v/v) Tween-20 (PBST) and incubated with 100 µL of avidin-conjugated alkaline phosphatase (1:3000) (Sigma) for 1 h at room temperature, and bound antibodies were visualized after washing six times with PBST and by addition of 50 µL of 1 mg/mL of *p*-nitrophenyl phosphate (*p*NPP) (Sigma) in 0.1 M diethanolamine buffer. The reaction was terminated after 20 min using 25 µL/well of 3 N NaOH.

Absorbance was read at 405 nm with an ELISA plate reader (Model 550, Bio-Rad Laboratories). The final absorbance values obtained were converted to concentration values by comparing the readings with a standard curve obtained from standard IgE samples ranging from 62.5 to 2000 ng IgE/mL. For detection of specific IgE, the plates were coated with 100 µL of 5 µg/mL of antigen diluted in 50 mM sodium bicarbonate (pH 9.6) and detection was carried out as described above, but with detection using serum and then anti-IgE; the results were expressed as absorbance at 405 nm.

Isolation of splenocytes

The spleen from individual mice was aseptically removed and mechanically released into ice-cold RPMI 1640 medium (Gibco) to release splenocytes. The cell suspensions were transferred to 15 mL conical centrifuge tubes and pelleted by centrifuging for 10 min at 500 g at 4 °C. Erythrocytes in spleen cell preparations were lysed with 5 mL of red blood cell (RBC) lysing buffer (Sigma) and 5 mL of RPMI media was added to stop the lysis. The splenocytes were washed twice with 10 mL RPMI 1640 by centrifugation. Splenocytes were resuspended in 5 mL of medium [RPMI 1640 supplemented with 8% fetal bovine serum (FBS)] (Hyclone, Fisher, Canada) and cell viability was determined by trypan blue exclusion and 1×10^6 cells/well were cultured in 96-well flat-bottom microwell plates (Corning Incorporated, Corning, NY, USA). Cultures in duplicate were stimulated with 50 µg of ovomucoid third domain DIII and P-Gly antigens and cells alone were used as unstimulated controls. After 72 h at 37 °C in a humidified 5% CO₂ incubator, the supernatants were collected and assayed for the presence of secreted cytokines and quantified along with standard cytokine samples.

Cytokine quantification

Levels of IL-4 and IFN-γ secreted in murine splenocyte culture supernatants of the spleen cells, in response to the DIII⁺ and P-Gly antigens, were measured after 72 h. IFN-γ and IL-4 were assayed using specific ELISA antibody sets (Pharmingen, San Diego, CA, USA). Microwell plates (Corning Incorporated) were coated with 100 µL of 2 µg/mL of rat anti-mouse IL-4 and INF-γ (BD Biosciences), in 0.1 M sodium phosphate buffer (pH 8.6). The plates were incubated at 4 °C overnight. The plates were washed three times with 200 µL of PBST and blocked with 200 µL of 2% BSA in PBS at 37 °C for 1 h. The plates were further washed three times with PBST and 100 µL of standard cytokines (BD Biosciences) at concentrations of 31.250–1000 pg/mL diluted in 2% (w/v) BSA in PBST, and the culture supernatant samples were added and incubated at 37 °C for 2 h. Hundred microlitres of 0.1 µg/mL of biotinylated rat anti-mouse IL-4 and INF-γ detec-

tion antibodies (BD Biosciences) were added and incubated at 37 °C for 2 h. Bound antibodies were detected using 100 µL of HRP-conjugated avidin (BD Biosciences) at 1 : 2000 dilution and the plate was incubated for 1 h at 37 °C and washed six times with PBST, and 50 µL of 3,3',5,5'-tetramethylbenzidine (TMB; Sigma) was used as a substrate and the plate was incubated for 20 min in the dark at 37 °C and 25 µL of stop solution (0.5 M H₂SO₄) was added. The optical density was measured by an ELISA Reader with a 450 nm filter. Standard curves for IL-4 and INF-γ were used to quantify the levels of cytokines present in the culture supernatant samples.

Immunoglobulin E-binding reactivity to ovomucoid third domain antigens

In order to evaluate, which among the two N-linked glycan sites (28 and 45) of the ovomucoid third domain was more involved in allergic reactions, we analysed the specific IgE-binding reactivity of the three ovomucoid DIII antigens using ELISA. As the glycosylation site at position 45 is the same site to which the carbohydrate chain is attached in the native ovomucoid, we investigated the immunosimilarity of the native DIII⁺ with carbohydrate, DIII without the carbohydrate (DIII⁻) and *Pichia*-expressed glycosylated ovomucoid third domain (P-Gly). IgE binding cross-reactivity to the DIII antigens of the ovomucoid was measured by an enzymatic immunoassay using a specific IgE protocol as described earlier. Briefly, micro titre plates were coated with 100 µL of 5 µg/mL of the carbohydrate containing native third domain (DIII⁺), without carbohydrate (DIII⁻) and recombinant glycosylated ovomucoid (P-Gly) third domain antigen, and incubated with 100 µL of serum samples (1 : 20 dilution) obtained from DIII⁺, DIII⁻ and P-Gly-sensitized mice. Biotinylated anti-mouse IgE (100 µL) (Caltag) (1 : 1000 dilutions) was added, followed by 100 µL of avidin-alkaline phosphatase (Sigma) (1 : 3000 dilution). The pNPP (1 mg/mL) (50 µL) was used for colour development and stopped with 25 µL of 3 N NaOH. The absorbance was read at 405 nm and the readings obtained were arbitrarily expressed as percentage binding index.

Carbohydrate detection

The total sugar contents of the samples were measured by the phenol-sulphuric acid test [20]. The carbohydrate content was calculated in mannose equivalents from a standard mannose curve. Briefly, the samples were dissolved in water in a test tube and to 100 µL of mannose or sample solution, 50 µL of 80% phenol (w/v) in sterile distilled water was added. Finally, 2.0 mL of concentrated sulphuric acid was added and the tubes were allowed to stand for 10 min at room temperature, then shaken and placed for 20 min in a water bath at 30 °C, and the

absorbance at 490 nm was measured against a water blank. The absorbance at 490 nm of a set of D-mannose standards ranging from 0–1000 µg was also read and a standard curve was constructed. The absorbance values of samples against standards were compared with calculate the total carbohydrate percentage content. Reported data expressed as percentage are means of duplicate analysis.

Statistical methods

All data were analysed, using the statistical software program from GraphPad InStat version 3.0 for Windows (GraphPad Software, San Diego, CA, USA). All data were expressed as means $\pm$ standard error of the mean (SEM). Comparisons between controls and treatment groups were evaluated by one-way analysis of variance (ANOVA). Statistical significant differences between various treatment groups were determined using post hoc comparison by Tukey–Kramer's multiple comparison tests using GraphPad software. Differences were considered to be statistically significant at P -value ≤ 0.05 .

Results

Purification and glycoprotein detection on sodium dodecyl sulphate-polyacrylamide gel electrophoresis and Western blot

After selection of the optimal conditions for the protein expression, the 'best producer' clone was used for the large-scale preparation. The transformed yeast culture containing the ovomucoid gene was grown at 30 °C for 3 days, which yielded the highest amount of protein and centrifuged. The supernatant from the medium of the culture was concentrated and exhaustively dialyzed against 20 mM Tris buffer (pH 8.0) to remove small metabolic contaminants from the yeast and then, for further purification of the recombinant protein, the sample was applied to the gel-filtration chromatography on a Superdex HR-75 column, which yielded about 10 mg/L of protein. Upon electrophoresis, a major band with an apparent molecular mass corresponding to 6 kDa was observed, which was confirmed to be a glycosylated form of ovomucoid by staining with PAS (Schiff's) stain as observed in the SDS-PAGE gel (Fig. 2). This also indicated that the preprosequence of the α -factor was correctly processed in the yeast system. The native ovomucoid third domain protein containing the carbohydrate chain was also purified to homogeneity as shown in Fig. 2. The presence of carbohydrates in ovomucoid third domain antigens was analysed by a reaction of the protein transferred onto a nitrocellulose membrane by Western blotting using biotin hydrazide coupled to the aldehyde groups generated in the sugar moiety of the glycoproteins,

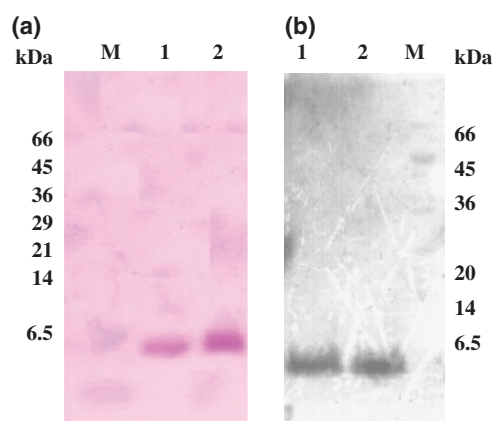


Fig. 2. SDS-PAGE analysis (a) and carbohydrate detection using Western Blot (b). (a) Active staining of the recombinant (P-Gly) and native (DIII⁺) ovomucoid antigens were performed using Periodic Acid-Schiff (PAS) stain. Cell pellets of *Pichia pastoris* transformed with ovomucoid were harvested as described in 'Materials and methods'. M represents the molecular weight markers. Lane 1 represents the recombinant ovomucoid third domain antigen (P-Gly) and lane 2 represents the native ovomucoid third domain antigen (DIII). (b) Western blot was developed with a streptavidin–biotin conjugate reaction as described in 'Materials and methods' to detect the presence of the glycoproteins. M represents biotinylated molecular weight markers. Lane 1 represents the recombinant glycosylated ovomucoid third domain antigen and lane 2 represents the native ovomucoid third domain antigen.

which also confirmed the existence of glycosylated forms of ovomucoid as shown in Fig. 2.

Carbohydrate content of glycosylated and native ovomucoid third domain

The carbohydrate moieties of ovomucoid present up to 20–25% of the total content of the intact ovomucoid molecule. When measured, the carbohydrate content of the glycosylated third domain (P-Gly) form and the native ovomucoid third domain (DIII⁺) antigens using the phenol–sulphuric acid assay yielded 16% of carbohydrate content in the native third domain antigen (DIII), and 13.8% in the recombinant glycosylated form of ovomucoid (P-Gly), when compared with a mannose standard curve (data are not shown).

Suppression of specific immunoglobulin E levels

Total and ovomucoid-specific IgE antibodies in immunized mice sera were determined by ELISA, which were raised against the glycosylated native (DIII) and recombinant glycosylated DIII (P-Gly) ovomucoid antigens. It was observed that the recombinant glycosylated form of ovomucoid (P-Gly) showed significantly reduced specific IgE levels (Fig. 3). This indicates a suppressive influence on IgE production, when compared with the native third domain of ovomucoid (DIII) antigen. Statistically no

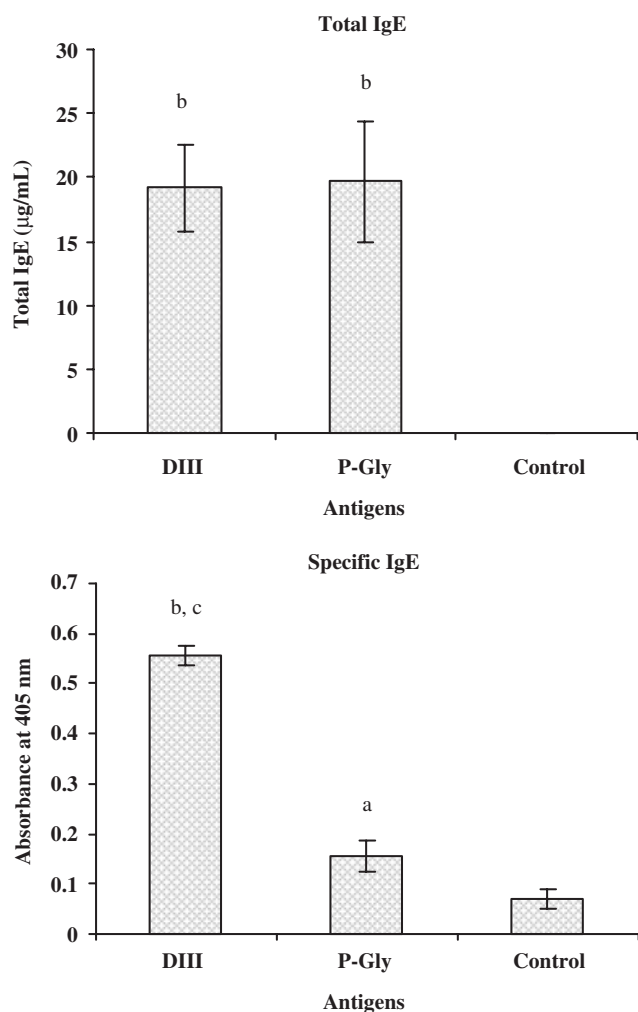


Fig. 3. Total and specific IgE binding of mice sera to recombinant and native ovomucoid third domain antigen. Immunochemical detection for total and specific IgE levels was determined using mice sera against the native and recombinant ovomucoid third domain antigens as described in 'Materials and methods' using ELISA. Total serum IgE levels are expressed as microgram per millilitre and ovomucoid-specific IgE levels are expressed as optical density (OD). The data were expressed as mean $\pm$ SEM from four individual mice ($n = 4$) (^a $P \leq 0.05$ vs. the control group; ^b $P \leq 0.001$ vs. the control group; ^c $P \leq 0.001$ vs. the P-Gly group).

difference was observed in the production of total IgE ($P > 0.05$). None or very little IgE was detected in the serum of mice in control groups immunized with PBS. Suppression of specific IgE levels ($P < 0.001$) in the glycosylated ovomucoid sera was indicative that the oligosaccharides (N-glycan moieties) inhibits allergic reactions by suppression of specific IgE levels, which confirms our earlier finding [6] that carbohydrates play an inhibitory role in allergic reactions of ovomucoid.

Alteration of T-helper type 1/type 2 cytokine ratios

It has been hypothesized that an alteration in the Th1/Th2 profile could play a role in regulating IgE responses

in vivo. To determine whether Th2/Th1 cytokine profiles were altered, we examined the cytokine levels in the culture supernatants of mice splenocytes stimulated with ovomucoid third domain antigens (DIII and P-Gly). Un-sensitized BALB/c mice were divided into three groups ($n = 4$ /group) and the first group was sensitized with DIII, the second group with P-Gly and the third group with PBS. Supernatants of allergen-stimulated spleen cells were analysed for cytokine levels. The administration of the recombinant ovomucoid profoundly affected the splenocyte production of the Th1 cytokine IFN- γ and of the Th2 cytokine IL-4 in mice. The production of a representative Th1 cytokine (IFN- γ) was found to be increased ($P < 0.05$), whereas the production of Th2 cytokine (IL-4) was significantly decreased ($P < 0.05$) with P-Gly, indicating a skewed Th1/Th2 balance in mice, biased towards a Th1 pathway. The down-regulation of the Th2 cytokine (IL-4) thereby results in the reduction of the Th2-mediated immunoglobulin, the IgE, which was significantly low in P-Gly serum samples, suggesting that the amounts of IgE produced depend on the relative quantity of IL-4 and IFN- γ generated by antigen-specific T cells. Increased IL-4 and specific IgE levels were observed with DIII and therefore cytokine regulation of antigen-driven immunoglobulin production is observed. Neither change of cell viability nor the number of isolated splenocytes was seen in the groups treated with P-Gly, suggesting that it did not modulate cell proliferation but differentiation. Therefore it could be concluded that P-Gly seems to stimulate Th1 cytokines, while decreasing Th2 cytokines as represented in Fig. 4, thereby showing inclination towards an anti-allergic Th1-skewed response and deviation from an expected Th2-allergic pathway.

Cross-reactivity of immunoglobulin E binding to ovomucoid third domain antigens

IgE antibody cross-reactivity to the three ovomucoid DIII antigens was investigated using an enzymatic immunoassay (ELISA) for specific IgE detection, to examine the levels of immunosimilarity in percentage binding of IgE between the ovomucoid DIII allergens (DIII⁺, DIII⁻ and P-Gly). Specific IgE-binding reactivity of three antigens of ovomucoid DIII was compared using antisera raised against the respective antigens (DIII⁺, DIII⁻ and P-Gly). The result indicated that sera raised against the ovomucoid third domain, with and without a carbohydrate chain (DIII⁺ and DIII⁻), did not show significant difference in IgE-binding activity between each other; furthermore, there was a high degree of cross-reactivity (i.e. anti-DIII⁺ recognizes DIII⁻ and vice versa), suggesting a high level of immunosimilarity. On the other hand, the immunosimilarity was low with P-Gly antigen against sera raised with and without the carbohydrate chain antigens ($P < 0.05$). Furthermore, IgE-binding reactivity in the DIII

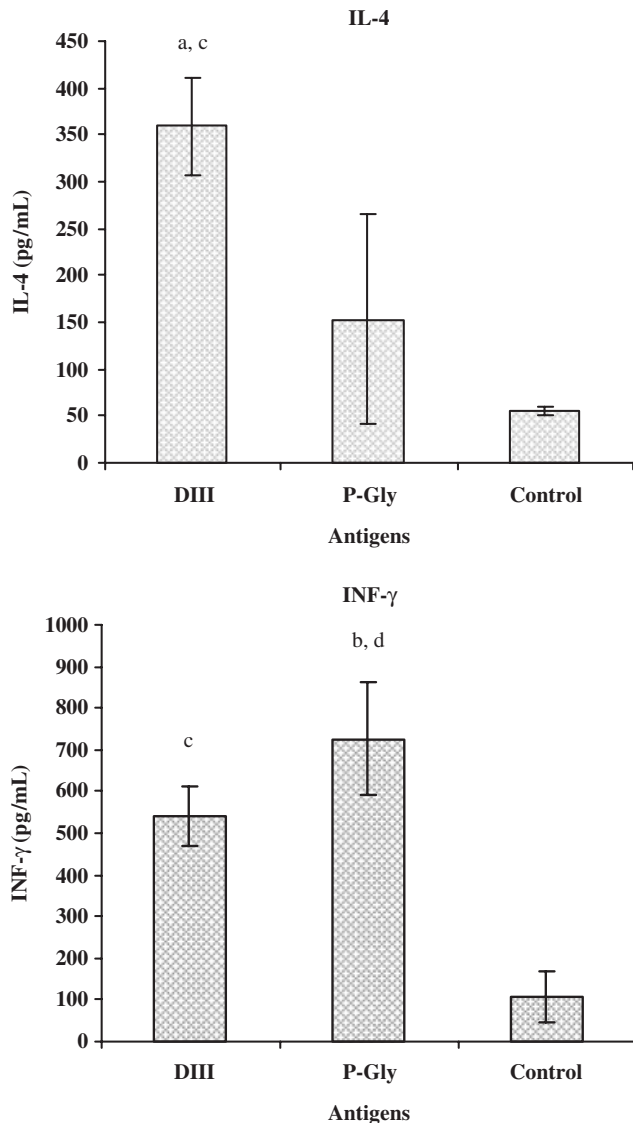


Fig. 4. Cytokine levels secreted by reactivated splenocytes from mice sensitized with native and recombinant glycosylated ovomucoid antigens. The spleen was excised from individual mice and splenocytes were isolated and reactivated with respective antigens and incubated for 3 days. At the end of 72 h, the supernatant was removed and quantified for IL-4 and INF- γ using standard cytokines. The data represented are mean $\pm$ SEM from four individual mice ($n = 4$) (^a $P \leq 0.05$ vs. the P-Gly group; ^b $P \leq 0.05$ vs. the DIII group; ^c $P \leq 0.01$ vs. the control group; ^d $P \leq 0.001$ vs. the control group).

antigens (with and without carbohydrate) against P-Gly sera indicated that the immunosimilarity between the DIII⁺ and DIII⁻ antigens is the same, with no difference in specific IgE binding with low binding reactivity. In contrast, P-Gly antigen was shown to have significantly high binding with high levels of specific IgE ($P < 0.05$), indicating thereby that the recognition of P-Gly antigen on ovomucoid third domain is different (Fig. 5). This result indicates that the cross-reactivity between DIII sera was induced by a similar, if not identical, allergenic epitope,

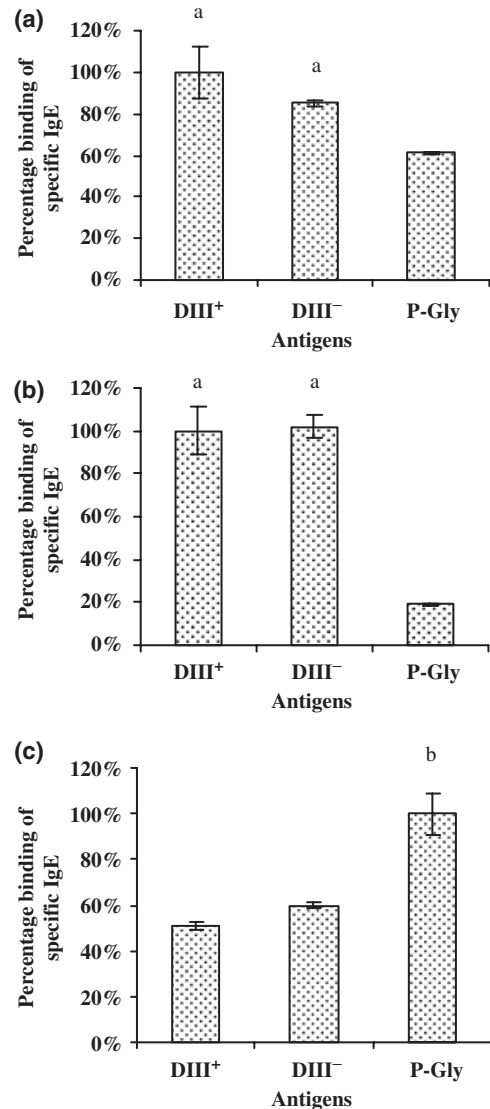


Fig. 5. Specific IgE binding percentage to ovomucoid DIII antigens was determined by ELISA. a, b and c panels represent the different antisera samples (a – antisera against DIII⁺ antigen; b – antisera against DIII⁻ antigen; c – antisera against P-Gly antigen) that were used to detect the specific IgE-binding reactivity. The levels of specific IgE are expressed as percentage binding levels. (^a $P \leq 0.05$ vs. the P-Gly-coated antigen group; ^b $P \leq 0.05$ vs. the DIII⁺ and DIII⁻ coated antigen group).

whereas the actual epitope is masked in case of P-Gly; thereby, the recognition pattern is different.

Therefore, it could be concluded that the carbohydrate chain at asparagine position 45 may not participate in an allergic reaction, whereas the N-glycan site at position 28 may play a role in the suppression of ovomucoid-induced allergic reactions. It can also be hypothesized that the suppression is associated with the position of the glycosylation site at N-glycan-28 (N28) on the three-dimensional structure of ovomucoid DIII. Indeed, the N28 seems to be masking the prime IgE-binding epitope region (α -helix) as shown in Fig. 6. Cross-reactivity is largely

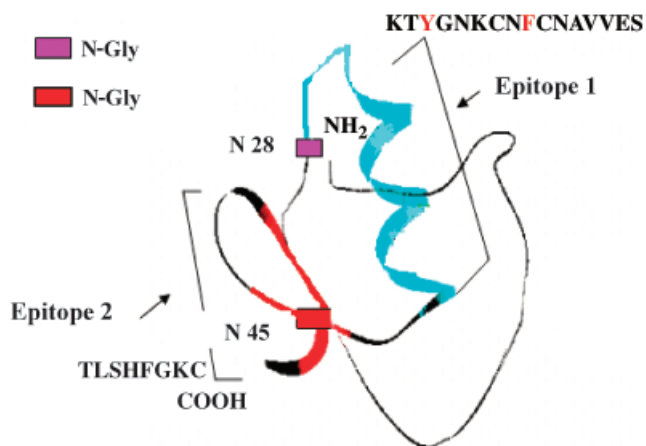


Fig. 6. Three-dimensional structural modelling of DIII ovomucoid. The solid ribbon structures represent the crystal structure. Side chains comprising the IgE binding epitopes are labelled. The box represents the location of the N-linked glycan (N-Gly) sites (adapted from www.pdb.org).

determined by structural aspects [21]. Antigens are cross-reactive only if they share structural features and are able to generate similar epitopes recognized by a set of antibodies generated during a polyclonal response. In this study, the native antigens seem to recognize a similar epitope and shows high similarity. However, in case of P-Gly the immunosimilarity is low when compared with other DIII antigens, due to its position on the protein. Thus, it was evident that the glycosylation site at position 28 on the ovomucoid molecule is involved in suppression of allergic reactions, whereas the site at position 45 did not seem to play a role in allergic reactions.

Discussion

The actual role of carbohydrate moieties on the allergenicity of ovomucoid still remains a controversy. Also, participation of N-linked glycan moieties in allergenicity of ovomucoid needs to be confirmed. Based on these assumptions, we hypothesized that constructing a glycosylated form of the third domain of ovomucoid and characterizing its role in the allergenicity of ovomucoid in a murine model would clarify the existing conflict. IgE binding epitopes of ovomucoid have been identified using egg-allergic patient's sera [22]. While the glycan moieties of ovomucoid are not a part of the IgE-binding epitopes identified, we evaluated the contribution of the glycan moieties of ovomucoid third domain to its allergenic potential, because both the N-linked glycan moieties in ovomucoid DIII lie adjacent to the epitopes identified, and could contribute to the stability of the protein, which could have an impact on their allergic function as mentioned earlier.

Recently, methylotrophic yeast *P. pastoris* has been developed as an excellent host for the large-scale expression of proteins from different sources [23, 24]. *Pichia pastoris* N-linked glycosylation consists mostly of short-chain mannose GlcNAc residues and is closer to the typical mammalian high-mannose glycosylation pattern. Detailed information on the structures of N-linked oligosaccharides in *P. pastoris* secreted proteins is of interest to many research groups [8] with regard to the functional and biological aspects of the antigen. In order to elucidate the structural and functional relationship of the glycosylated ovomucoid third domain in relation to its allergenic activity and to analyse the critical factor for IgE-binding strength of the N-linked glycan moieties, we investigated the expression of ovomucoid DIII in yeast *P. pastoris* system.

Carbohydrates have been shown to have an inhibitory effect on the T cell response and inhibited IL-4 production [4], but whether carbohydrates directly participate in allergic reactions is still under investigation. Suppression of an established IgE antibody response against ovomucoid-induced allergic reactions requires attention due to the fact that allergy to egg is frequent in children [25]. We have earlier shown that carbohydrate moieties in the third domain of ovomucoid had a rather inhibiting effect on its IgG- and IgE-binding activities [6]. In contrast, it has also been shown that carbohydrate moieties seem to have an effect on allergic reactions caused by ovomucoid [4, 26]. Cooke and Sampson [27] have reported that carbohydrate moieties have a minor effect on allergenicity of ovomucoid. The clinical relevance of such carbohydrates or N-glycans as IgE-binding epitopes is still under debate.

Structural studies on the carbohydrate moiety of hen ovomucoid have been reported earlier [28], but the effect on the *in vivo* response and the role of N-linked glycan moieties in allergenicity of ovomucoid has not been studied. We have determined the carbohydrate content of the native third domain of ovomucoid to be 16% and the glycosylated form as 13.8% and the whole ovomucoid carbohydrate content has been earlier reported to be 20–25% [1]. Comparison of the allergenic activity between the native ovomucoid third domain antigen (with carbohydrate chain) (DIII) and recombinant glycosylated ovomucoid antigen (P-Gly) revealed that specific IgE molecules seemed sterically hindered from binding to the allergen in case of P-Gly, whereas DIII indicated high levels of specific IgE levels. Thus, it could be concluded that the suppression effect is related to hindrance of the mannose chain occurring from glycosylation rather than the entire carbohydrate chain associated with the ovomucoid third domain.

IgE is elevated by a Th2-type response and as it was found that IgE levels are suppressed in P-Gly sera, the immune response was modulated to a Th1-dominant response. The IgE-binding epitopes recognized by allergic

patients and mice sera do not contain the glycan sites, although the significant difference in inhibitory potency could point to a contribution to antibody binding of the peptide regions in the neighbourhood of the N-glycan, as the N-glycan site at position 28 lies adjacent to the dominant IgE-binding epitope.

Conclusion

The inference from the above study is that the N-linked glycan (N28) in the third domain of ovomucoid seemed to have contributed to a suppressive effect on the allergenicity of the third domain of ovomucoid and as the pathogenesis of any allergic reactions is exclusively defined by the ability to bind IgE, it is noteworthy to consider the suppressive effect of P-Gly for immunotherapeutic treatment options and as a potential candidate for speculation of a hyposensitized target for treatment against egg-induced allergic reactions.

Acknowledgements

This work was supported by a grant from Natural Sciences and Research Council of Canada (NSERC) under a discovery grant programme.

Author's contributions: P. R. performed the experiments relating to all animal experiments, immunological data and drafted the manuscript, Y. M. participated in its design, supervision and coordination and S. N. participated in the molecular cloning of the glycosylated ovomucoid. All authors read and approved the final manuscript.

References

- Kato I, Schrodde J, Kohr WJ, Laskowski M Jr. Chicken ovomucoid: determination of its amino acid sequence, determination of the trypsin reactive site and preparation of all three of its domains. *Biochemistry* 1987; 26:193–201.
- Yet MG, Chin CC, Wold F. The covalent structure of individual N-linked glycopeptides from ovomucoid and asialofetuin. *J Biol Chem* 1988; 263:111–7.
- Beeley JG. The isolation of ovomucoid variants differing in carbohydrate composition. *Biochem J* 1971; 123:399–405.
- Matsuda T, Nakamura R, Nakashima I, Hasegawa Y, Shimokata K. Human IgE antibody to the carbohydrate-containing third domain of chicken ovomucoid. *Biochem Biophys Res Commun* 1985; 129:505–10.
- Besler M, Steinhart H, Paschke A. Allergenicity of hen's egg-white proteins: IgE binding of native and deglycosylated ovomucoid. *Food Agric Immunol* 1997; 9:277–88.
- Zhang JW, Mine Y. Characterization of IgE and IgG epitopes on ovomucoid using egg white allergic patient's sera. *Biochem Biophys Res Commun* 1998; 253:124–7.
- Lauer I, Foetisch K, Kolarich D *et al.* Hazelnut (*Corylus avellana*) vicilin Cor a 11: molecular characterization of a glycoprotein and its allergenic activity. *Biochem J* 2004; 383:327–34.
- Calabozo B, Barber D, Polo F. Studies on the carbohydrate moiety of Pla 1 1 allergen. Identification of a major N-glycan and significance for the immunoglobulin E-binding activity. *Clin Exp Allergy* 2002; 32:1628–34.
- Okano M, Kino K, Takishita T *et al.* Roles of carbohydrates on Cry j 1, the major allergen of Japanese cedar pollen, in specific T-cell responses. *J Allergy Clin Immunol* 2001; 108:101–8.
- Petersen A, Schramm G, Schlaak M, Becker WM. Post-translational modifications influence IgE reactivity to the major allergen Phl p 1 of timothy grass pollen. *Clin Exp Allergy* 1998; 28:315–21.
- Nakamura S, Hata J, Kawamukai M *et al.* Enhanced anti-rotavirus action of human cystatin C by site-specific glycosylation in yeast. *Bioconjugate Chem* 2004; 15:1289–96.
- Cregg J, Vedvick TS, Raschke WC. Recent advances in the expression of foreign genes in *Pichia pastoris*. *Biotechnology* 1993; 11:905–10.
- White CE, Kemp NM, Komives EA. Expression of highly disulphide-bonded proteins in *Pichia pastoris*. *Structure* 1994; 2:1003–5.
- Clare JJ, Rayment FB, Ballantine SP, Sreekrishna K, Ramanos MA. High-level expression of tetanus toxin fragment C in *Pichia pastoris* strains containing multiple tandem integrations of the gene. *Biotechnology* 1991; 9:455–60.
- Ikegaya K, Hirose M, Ohmura T, Nokihara K. Complete determination of disulfide forms of purified recombinant human serum albumin, secreted by the yeast *Pichia pastoris*. *Anal Chem* 1997; 69:1986–91.
- Liu ST, Saito A, Azakami H, Kato A. Expression, purification, and characterization of an unstable lysozyme mutant in *Pichia pastoris*. *Protein Exp Purif* 2003; 27:304–12.
- Waterham HR, Digan ME, Koutz PJ, Lair SV, Cregg JM. Isolation of the *Pichia pastoris* glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter. *Gene* 1997; 186:37–44.
- Burnette WN. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 1981; 112:195–203.
- Kondo M, Harada H, Sunada S, Yamaguchi T. Increased selectivity in the detection of glycoproteins on nitrocellulose membranes by washing with sodium hydroxide solution. *Electrophoresis* 1991; 12:685–6.
- Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. Colorimetric method for determination of sugars and related substances. *Anal Chem* 1956; 28:350–6.
- Aalberse RC. Structural biology of allergens. *J Allergy Clin Immunol* 2000; 106:228–38.
- Mine Y, Zhang JW. Identification and fine mapping of IgG and IgE epitopes in ovomucoid. *Biochem Biophys Res Commun* 2002; 292:1070–4.
- Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol Rev* 2000; 24:45–66.
- Schuster M, Einhauer A, Wasserbauer E *et al.* Protein expression in yeast; comparison of two expression strategies regarding protein maturation. *J Biotechnol* 2000; 84:237–48.

- 25 Eggesbo M, Botten G, Halvorsen R, Magnus P. The prevalence of allergy to egg: a population-based study in young children. *Allergy* 2001; **56**:403–11.
- 26 Matsuda T, Nakashima I, Nakamura R, Shimokata K. Specificity to ovomucoid domains of human serum antibody from allergic patients: comparison with anti-ovomucoid antibody from laboratory animals. *J Biochem* 1986; **100**:985–8.
- 27 Cooke SK, Sampson HA. Allergenic properties of ovomucoid in man. *J Immunol* 1997; **159**:2026–32.
- 28 Yamashita K, Kammerling JP, Kobata A. Structural studies of the sugar chains of hen ovomucoid. Evidence indicating that they are formed mainly by the alternate biosynthetic pathway of asparagine-linked sugar chains. *J Biol Chem* 1982; **257**:12809–14.