

Substitution of *Pichia pastoris*-Derived Recombinant Proteins with Mannose Containing O- and N-Linked Glycans Decreases Specificity of Diagnostic Tests

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Key Words

Pichia pastoris · Recombinant proteins · Mannose · O- and N-linked glycans · Cross-reactivity

Abstract

Background: Recombinant proteins from *Pichia pastoris* need to be fully evaluated before used as diagnostic tools. **Objective:** The objective of this study was to investigate whether glycosylation by *P. pastoris* interferes with the specificity of diagnostic tests. **Methods:** An autoantigen involved in Wegener's disease (protease 3) and 2 major inhalant allergens from grass pollen (Dac g 5) and house dust mite (Der p 1) were produced as recombinant molecules in *P. pastoris*. O-linked glycans on Dac g 5 were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The immune reactivity of the recombinant proteins was compared to that of their natural counterparts by ELISA and a radio-allergosorbent test (RAST) as well as by ELISA and RAST inhibition. **Results:** In contrast to the non-glycosylated natural allergen, recombinant Dac g 5 was shown to carry at least 2 small mannose-containing O-glycans. We showed that both these O-glycans and the

N-linked glycans on recombinant protease 3 and recombinant Der p 1 were recognized in ELISA by IgG antibodies in sera of healthy individuals. These IgG responses were closely correlated. The natural autoantigen and allergens were not recognized by IgG antibodies from healthy subjects. The carbohydrate nature of the epitopes recognized by IgG on the recombinant proteins was confirmed by inhibition studies with mannose and yeast mannan. IgE recognition of yeast glycans was observed in 2 out of 9 positive sera from patients with allergic bronchopulmonary aspergillosis. **Conclusion:** Production of recombinant molecules in yeast (or moulds) can introduce IgG-binding glycans that negatively affect the specificity of diagnostic tests.

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Introduction

Recombinant technology has revolutionized the development of diagnostic tests and the production of therapeutic proteins. It allows the production of disease-related molecules in reproducible fashion and large quantities. In the late 1980s, the production of recombinant proteins

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was largely limited to prokaryotic expression systems like *Escherichia coli*. Since the machinery of prokaryotic organisms for post-translational modifications is limited, eukaryotic expression systems have rapidly been introduced next to *E. coli*. These include yeasts and moulds, insect cells, mammalian cells and plants. Yeast expression systems like *Saccharomyces cerevisiae* and *Pichia pastoris* combine the advantages of rapid and easy molecular manipulation with the ability to introduce post-translational modifications such as glycosylation and efficient formation of disulfide bonds. Glycosylation is often essential for the conformation and stability of the proteins, but at the same time yeast glycans might have antigenic, immunogenic and immune-modulating properties for mammals [1–4]. Antigenicity of recombinant yeast glycoproteins might therefore affect the specificity of diagnostic tests, whereas immunogenicity and immune-modulating properties may be relevant in relation to their behavior as therapeutic molecules. Yeast-derived mannans have been shown to bind to serum proteins like mannan-binding proteins [5]: upon binding, the glycan structures may be able to activate the innate immune system via the mannan-binding pathway.

S. cerevisiae was the first yeast used for the production of recombinant proteins, but *P. pastoris* is used far more frequently at present. One of the reasons why the interest in *S. cerevisiae* has diminished is its tendency to hyperglycosylate. Proteins expressed in *S. cerevisiae* are often modified by substitution with N-linked glycans with a core structure harboring at least 9–13 mannose residues (Man_{9–13}GlcNAc₂), so-called high-mannose glycans. However, extensions of outer chains with up to 200 mannose residues may occur [6]. Usually, these chains are linked to the α -1,3-branch on the core, but in some cases short α -1,2-linked mannose chains are attached. *P. pastoris* is generally regarded to produce smaller high-mannose N-glycans. In both *S. cerevisiae* and *P. pastoris*, charged groups can be linked to N-glycans in the form of mannose phosphate diesters [7–9]. Yeast can also substitute serines and threonines with O-linked glycans. O-glycosylation begins in the endoplasmic reticulum by addition of a single mannose. After transportation to the Golgi, several α -(1,2)-linked mannoses can be attached that may finally be capped with one or two α -(1,3)-linked mannoses [10].

Specific antibodies to yeast glycans have been shown to be directed to side chains of yeast carbohydrate moieties. Such antibodies were, for example, described against tri- and tetrasaccharide side chains of glycans found in the yeast *Saccharomyces lactis* [11]. In *S. cerevisiae*, a 3-unit structure containing (1,3),(1,2),(1,2)-linked α -D-manno-

pyranose was shown to be an antigenic structure [12]. Recently, we produced an autoantigen and 2 inhalant allergens in *P. pastoris* for application in diagnostic tests. The auto-antigen, protease 3 (Pr3), involved in Wegener's disease [13], has a putative N-glycosylation site and was found to be glycosylated by the yeast. The recombinant molecule binds Pr3-specific IgG similarly to the natural protein. The major house dust mite allergen from *Dermaphagoides pteronyssinus*, Der p 1, is a cysteine protease with two consensus sequences for N-glycosylation, one in the pro-sequence of the pro-enzyme and one in the mature enzyme [14]. There is some controversy about whether, and if so at which position, the natural allergen is glycosylated [15, 16]. The allergen was shown to be glycosylated upon expression in *P. pastoris* and was reported to have IgE reactivity similar to its natural counterpart when testing sera of house dust mite allergic patients [17]. Unexpectedly, the major *Dactylis glomerata* grass pollen allergen, Dac g 5, was also glycosylated by this yeast. The primary sequence of Dac g 5 does not harbor any consensus sequences for N-glycosylation, and the natural allergen is not glycosylated. Most likely, the allergen is substituted with O-glycans during expression in *P. pastoris*. Despite these differences between natural and recombinant Dac g 5, both molecules displayed very similar IgE-binding characteristics when sera from patients allergic to grass pollen were tested [18].

The aim of our study was to investigate the structure of the O-linked glycan(s) on rDac g 5, and to examine whether yeast glycans influence the performance of immunoassays for the diagnosis of autoimmune and allergic diseases.

Material and Methods

Production of Recombinant Proteins

Recombinant Dac g 5 (rDac g 5) and proDer p 1 (rproDer p 1) were expressed in *P. pastoris* as described earlier [17, 18]. In short, cDNAs coding for rDac g 5 and rproDer p 1 were cloned in the pPICZ α A vector, facilitating secretion into the medium. Transformation into the *P. pastoris* strain, SMD1168H, was carried out as prescribed by the manufacturer (Invitrogen, San Diego, Calif., USA). The positive clones grown under zeocine selection in yeast extract peptone dextrose medium (YPD) were transferred to buffered glycerol-complex medium (BMGY) in culture flasks at 29°C for 24 h. Glycerol was removed by centrifugation, and subsequently the cells were inoculated in buffered methanol-complex medium at OD_{600nm} 10 for induction of expression. After 4–5 days, the supernatants were harvested by centrifugation. For the expression of enzymatically inactive recombinant Pr3 (rPr3), its active site serine (Ser 176) was changed to an alanine using the overlap extension method [19]. The resulting cDNA was sequenced and subcloned into the *P. pastoris*

expression vector pPicZaA, which was subsequently transformed to the *P. pastoris* strain KM71, according to the manufacturer's instructions (Invitrogen). A Pr3-secreting clone was cultured at pH 5.0 in a Bioflo 3000 fermentor (New Brunswick Scientific, Nijmegen, The Netherlands), in minimal basal salt medium supplemented with glycerol, histidine, biotin and 0.2 % (v/v) PMT1 trace salts (Invitrogen). Fermentation proceeded in 2 steps: first, to begin the initial 'glycerol phase', 250 ml of start-up culture (cells grown in flasks in BMGY medium) was added to the fermentor containing 3 liters of medium. The yeasts were grown at 30 °C and were agitated at 500 rpm. A pH of 5.0 was maintained using 30% (w/v) ammonium hydroxide, and pure oxygen was supplied to keep the dissolved oxygen at or above 30%. After all the glycerol was consumed (30 h), the synthesis of Pr3 was induced by adding methanol. After 7 days of culture, at a cell wet weight of 160 g/l, the yeasts were harvested and the cells were pelleted by centrifugation. Then the supernatant was concentrated and dialyzed at the same time against PBS (pH 7.4) by using a capillary dialyzer (Hemoflo F5, Fresenius, Bad Homburg, Germany). Preceding affinity purification of rPr3 from dialyzed and concentrated yeast culture supernatant, Triton X-100 was added to a final concentration of 0.05% (v/v).

Purification of Recombinant and Natural Proteins

Natural Dac g 5 (nDac g 5) was purified from a *D. glomerata* grass pollen extract by affinity chromatography with a monoclonal antibody (mAb) (4B1) coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) [20]. After the protein was allowed to bind, the column was washed with PBS and subsequently eluted with 0.1 mol/l glycine (pH 2.5). After dialysis against PBS, the samples were stored at -20 °C until further use. Natural Der p 1 (nDer p 1) was purified from a spent culture medium extract (CSL, Melbourne, Australia) using a Sepharose-coupled mAb directed to Der p 1 [21]. Elution was performed with 50% ethylene glycol/5 mmol/l lysine (pH 11). rDac g 5 and rproDer p 1 were purified from yeast culture supernatants by the same method as described for their natural counterparts.

Affinity purification was performed by an anti-Pr3 mAb 12.8 [22] coupled to Sepharose. Bound protein was eluted with a 5 mmol/l lysine buffer (pH 11.5) containing 0.05% Triton X-100.

The purity of all three recombinant molecules was assessed by SDS-PAGE/silver staining (Novex, San Diego, Calif., USA). Protein concentrations were determined using the BCA method prescribed by the manufacturer (Pierce, Rockford, Ill., USA).

SDS-PAGE/Silver Staining and Glycan Detection by ConA Binding

Affinity-purified allergens were separated by SDS-PAGE on 4–12% gradient gels (Novex) as prescribed by the manufacturer, and silver-stained according to the Bixel Biotechnology silver-staining kit protocol (Bixel Biotechnology, Union City, Calif., USA). Affinity-purified rPr3 was separated by SDS-PAGE on 8–18% ExcelGel and silver-stained according to the manufacturer's instructions (Amersham Pharmacia Biotech AB).

Purified natural and recombinant proteins were electroblotted onto nitrocellulose membrane followed by an overnight incubation in Tris-buffered saline/0.1% Tween-20 (TBS-T). Subsequently, the blot was incubated with ConA (25 µg/ml) (Sigma, St. Louis, Mo., USA) in TBS-T/1 mmol/l MgCl₂/1 mmol/l CaCl₂ for 90 min. After washing with the same buffer, the membrane was incubated with 50 µg horseradish peroxidase (HRP; Sigma) for 60 min [23]. Glyco-

proteins were visualized with 1 tablet of DAB in 40 ml H₂O (10 mg diaminobenzidine-tetrahydrochloride; Kem-En-Tec, Copenhagen, Denmark). The reaction was started with 40 µl of 30% H₂O₂ and ended by washing the blots in H₂O.

Separation of rDac g 5 by 2D Gel Electrophoresis

Fifty micrograms of rDac g 5 were dissolved in isoelectric-focusing buffer (final volume, 400 µl) consisting of 5 mol/l urea, 2 mol/l thio-urea, 33 mmol/l 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate, 2 mmol/l tributyl phosphine, 10 mmol/l dithiothreitol, 2% (v/v) carrier ampholytes (pH 3.5–10; Sigma), and 0.4% (w/v) bromophenol blue. The first-dimension gel separation was carried out with Immobiline Dry Strips NL (pH 3–10; Amersham Pharmacia Biotech AB, Uppsala, Sweden). The second-dimension separation was obtained by SDS-PAGE with a 12.5% (w/v) polyacrylamide resolving gel (width 16 cm, length 20 cm, thickness 0.75 mm).

Tryptic Digestion and Isolation of Glycopeptides from rDac g 5

Fifty micrograms of rDac g 5 were run on 15% SDS-PAGE and subsequently stained with Coomassie Brilliant Blue. The protein band corresponding to the recombinant allergen was excised and cut into small fragments. The fragments were washed 3 times with 100 µl of a solution of 0.1 mol/l NH₄HCO₃/CH₃CN (1/1) for 15 min each time and dried out. Protein was then digested with 7.5 µg of trypsin in 200 µl of 0.05 mol/l NH₄HCO₃ at 37 °C for 16 h. Two hundred microliters of CH₃CN were added and the supernatant was collected. Gel fragments were then washed with 50 µl of 0.1 mol/l NH₄HCO₃, then again with 50 µl CH₃CN, and finally with 50 µl 5% formic acid. All supernatants were pooled and lyophilized. Peptide separation by HPLC was carried out on a C₁₈ reverse-phase column (4.5 × 250 mm) with a linear gradient of CH₃CN in TFA 0.1%. Fractions were collected, lyophilized and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

MALDI-TOF MS

MALDI-TOF mass spectra were measured on a Micromass Tof. Spec. E. MALDI-TOF mass spectrometer (Manchester, UK) equipped with a nitrogen laser operating at 337 nm. Mass spectra were performed in positive ion mode using α -cyano-4-hydroxycinnamic acid (10 mg/ml) as matrix. Each of the samples (2 µl) was mixed with 2 µl of the matrix solution in TFA:CH₃CN (1.75:0.75; v/v). Two microliters of this solution was applied onto a stainless steel sample plate and allowed to dry under vacuum. Spectra were acquired in the reflectron mode.

ELISA for Detection of Specific IgG Antibodies

Natural and recombinant allergens were coated overnight at 4 °C to microtiter plates (Maxisorb, Nunc, Roskilde, Denmark) at a concentration of 0.25 µg/100 µl coating buffer (0.1 mol/l NaHCO₃/pH 8.5). Nonspecific binding was decreased by application of high-performance ELISA buffer (Sanquin, Amsterdam, The Netherlands) for dilution of the sera (1:30–1:800) and of the HRP-labeled conjugate goat anti-human IgG antibody (1:20,000) (Sigma). Color development was initiated by adding 100 µl of 10 mg/ml TMB (3,3', 5,5'-tetramethylbenzidine) in sodium acetate, pH 5.5 and 10 µl 3% H₂O₂. The reaction was stopped by adding 2 M H₂SO₄, after which the absorbance was measured at 450 and 540 nm.

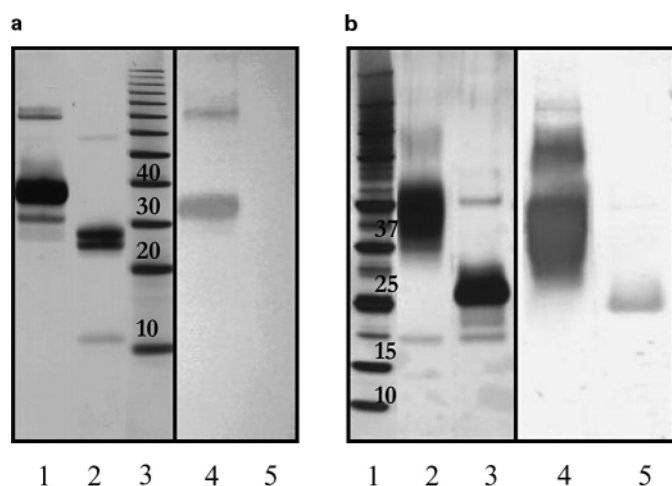


Fig. 1. SDS-PAGE/silver staining and ConA binding. **a** Lanes 1–3 show SDS-PAGE/silver staining of affinity-purified rDac g 5.01, nDac g 5 and a 10-kDa protein ladder (Life Technologies), respectively. Positive and negative binding to ConA of rDac g 5 and nDac g 5 are shown in lanes 4 and 5, respectively. **b** Lanes 1–3 show a SDS-PAGE/silver staining of a precision protein ladder (Biorad), rproDer p 1 and affinity-purified nDer p 1, respectively. Lanes 4 and 5 represent the results obtained by ConA binding for rproDer p 1 and nDer p 1, respectively.

ELISA inhibition experiments were performed by preincubation of serum with 2-O- α -D-mannopyranosyl-D-mannopyranose, 3-O- α -D-mannopyranosyl-D-mannopyranose or α -(1,3), α -(1,6)-mannotriose- α -methyl-glycoside (Sigma) (0–16 mM), prior to adding to allergen-coated wells.

Quantification of Pr3-Specific IgG Autoantibodies

For detection of Pr3-specific IgG autoantibodies, Pr3-specific mAb 12.8 [22] was coated to microtiter plates and used to capture *P. pastoris*-derived rPr3 or natural Pr3 from PMN lysates [22]. Wells were then incubated for 1 h at room temperature with human sera, diluted 1:20, 1:80, 1:320 and 1:1,280 in a buffer containing 20 mmol/l Tris-HCl (pH 6.8)/0.3 mol/l NaCl/0.25% Tween-20/1.0% BSA/5.0% normal goat serum. Bound IgG was detected by HRP-conjugated mAb against human IgG (Sanquin). Binding was expressed in arbitrary units, using a pool of Pr3-positive antineutrophil cytoplasmic autoantibody sera (set at 10,000 AU/ml) for construction of a standard curve.

RAST and RAST Inhibition

A radio-allergosorbent test (RAST) was performed as described previously [24]. Briefly, both natural and recombinant allergens were coupled to CNBr-activated Sepharose 4B at 100 μ g/100 mg Sepharose. Human serum samples (50 μ l) were incubated with 0.5 mg Sepharose in a final volume of 300 μ l PBS/0.3% BSA/0.1% Tween. After overnight incubation, unbound material was washed away and 50 μ l of ^{125} I-labeled sheep antihuman IgE (SH25; kindly provided by S.O. Stapel, Sanquin) was added. After overnight incubation and washing, radioactivity was measured in a gamma-counter. Results were expressed in international units/ml (IU/ml) IgE,

which were calculated using a standard curve of a human/mouse chimeric IgE antibody directed to Der p 2 and Sepharose-coupled rDer p 2 [25]. A result greater than 0.30 IU/ml was regarded as positive.

For RAST inhibition [24], preincubation of a human serum with 50 μ l rDac g 5 (55 μ g/ml), nDac g 5 (7.5 μ g/ml) and *Aspergillus fumigatus* mould extract (9 mg/ml) was allowed prior to adding Sepharose-coupled rDac g 5. Samples were further treated as described for the RAST.

Sera

Sera of healthy volunteers (n = 39) (blood bank donors, not screened for IgE or IgG against grass pollen or house dust mite allergens) were used to evaluate the specificity of ELISA for the measurement of IgG antibodies against yeast-derived glycoproteins (rDac g 5, rproDer p 1 and rPr3). In the case of rPr3, sera of 6 patients with Wegener's disease were used as positive controls.

To evaluate the specificity of rDac g 5 and rproDer p 1 RAST, sera of 9 patients with allergic bronchopulmonary aspergillosis (ABPA) were tested.

Results

Production of Purified Natural and Recombinant Proteins

The major grass pollen group 5 allergen, Dac g 5 [18], the pro-form of the major house dust mite group 1 allergen, Der p 1 [17], and the autoantigen Pr3 were successfully expressed in the yeast *P. pastoris* and affinity-purified from culture supernatants. Natural homologues of Dac g 5 and Der p 1 were isolated from *D. glomerata* grass pollen extract and from *D. pteronyssinus* house dust mite extract, respectively. SDS-PAGE/silver-staining analysis revealed that rDac g 5 (fig. 1a) and rproDer p 1 (fig. 1b) migrated at significantly higher apparent M_r than their natural counterparts and were both detected with ConA. Natural Dac g 5 was not detected by ConA, whereas nDer p 1 was only weakly stained.

MALDI-TOF MS

Gas chromatographic analysis of hydrolyzed rDac g 5 demonstrated the presence of mannose exclusively (not shown). To further analyze the nature of glycan moieties on rDac g 5, the allergen was separated by 2D SDS-PAGE into 3 distinct spots, each of which was digested in-gel with trypsin. Resulting tryptic peptides were analyzed by MALDI-TOF MS. The tryptic peptides detected from all 3 spots were identical and covered most of the known sequence of Dac g 5. The N- and C-terminal peptides were not identified with this method (fig. 2a). In addition, glycopeptides were not detected in any of these 3 digests. This was probably due to the competition in the desorp-

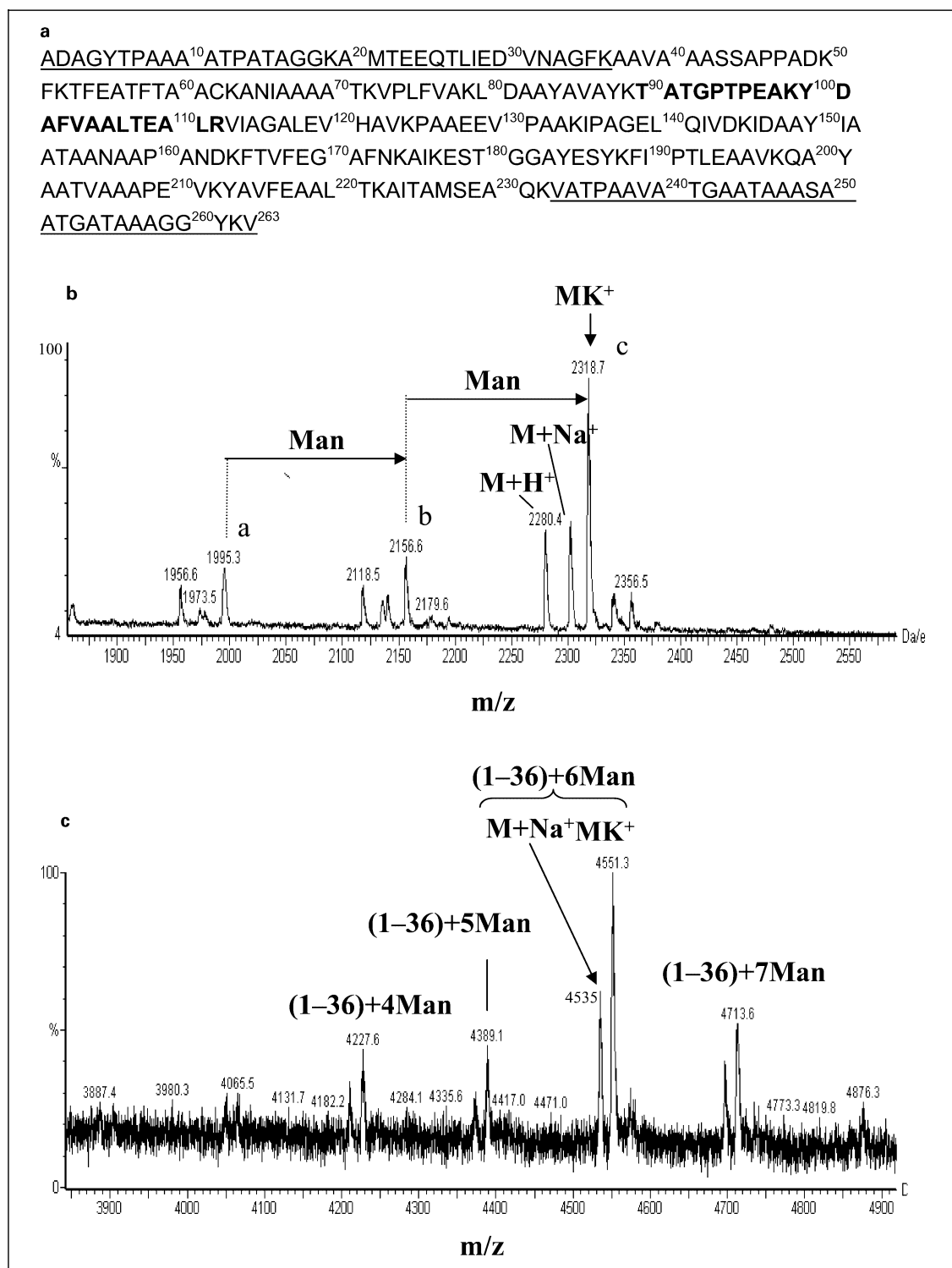


Fig. 2. MALDI-TOF MS of peptides and glycopeptides isolated from rDac g 5. **a** Sequence of rDac g 5 marking the peptides detected by MALDI-TOF MS. Underlined N- and C-terminal peptides were not detected after 2D gel experiments. The sequence in bold could not be identified after 1D gel experiments. The rest of the sequence was detected in both 1D and 2D gel experiments. **b** Showing peptides

(including a potassium ion) with M_r : 1,995 (peak a), 2,156 (peak b) and 2,318 Da (peak c), differing by 1 and 2 mannose residues. The x-axis represents the mass-to-charge ratios (m/z). The y-axis represents the ion intensity in percentage. **c** Peptide 1–36 M_r 3,540 Da was found, mixed with 4 peptides of which the M_r is in agreement with substitutions of 4, 5, 6 or 7 mannose residues.

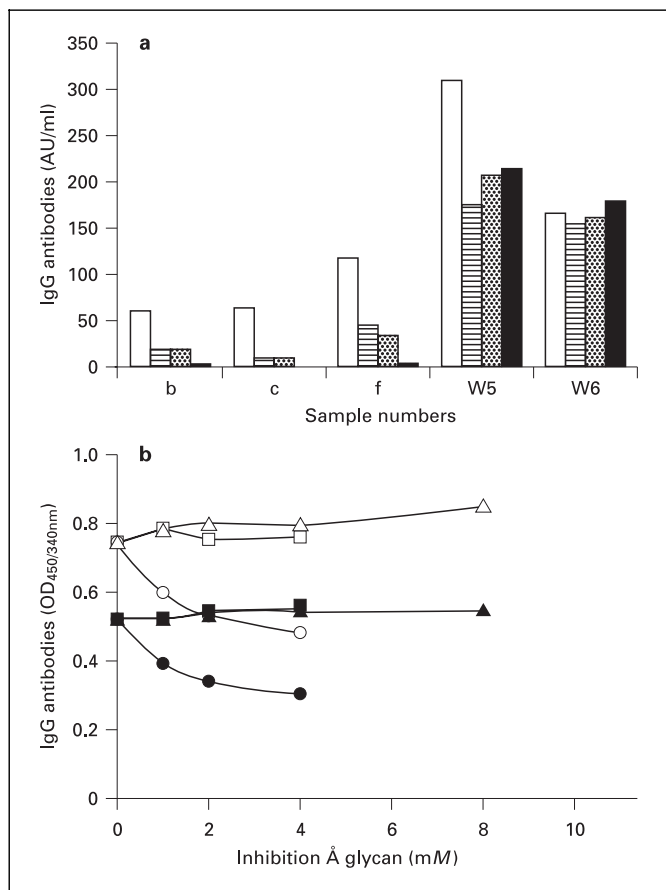


Fig. 3. ELISA for detection of anti-Pr3 IgG autoantibodies. **a** The white and black bars represent the responses of healthy controls (b, c, f) and Wegener's disease patients (W5, W6) towards rPr3 (□) and nPr3 (■), respectively. Preincubation with 250 µg/ml mannan (▨) or 50 mM mannose (▩) almost completely inhibits binding of IgG to rPr3 in sera of healthy controls in contrast to Wegener's sera. **b** Two healthy controls, p15 (○, △, □) and p17 (●, ▲, ●), were pre-incubated with 2-O- α -D-mannopyranosyl-D-mannopyranose (△, ▲), 3-O- α -D-mannopyranosyl-D-mannopyranose (□, ■) and α -(1,3), α -(1,6)-mannotriose- α -methyl-glycoside (○, ●), respectively.

tion of ions which make glycopeptides difficult to detect when analyzed in a mixture with non-glycopeptides. As a consequence, rDac g 5 was then separated by 1D SDS-PAGE and the resulting protein band was digested in-gel with trypsin. Tryptic peptides were isolated by reverse-phase HPLC and individually analyzed by MALDI-TOF MS. The same tryptic peptides as found after 2D SDS-PAGE were present again. In addition, one HPLC peak was shown to contain a mixture of M (mass) + H⁺, M + Na⁺ and M + K⁺ ions of 3 molecular species (fig. 2b, peaks a–c). The mass of these peptides could not be matched to

any pepsine-generated peptide based on the reported Dac g 5 sequence. However, the mass increments between the smallest (peak a) and the larger two peptides (peak b, c) were 162 and 324 Da, respectively. This agrees exactly with the mass of 1 and 2 mannose residues. Finally, in the same HPLC fraction, M + K⁺ ions differing by 162 Da were also detected in the 4,000- to 5,000-Da mass range and were assigned to a peptide corresponding to the 36 N-terminal amino acids. The mass of this peptide is 3,540 Da, substitutions of 4, 5, 6 or 7 mannose residues (each 162 Da), and the addition of a potassium ion (39 Da) or sodium ion (23 Da) will correspond to peaks representing glycopeptides with masses of 4,227, 4,389, 4,551 and 4,713 Da, respectively (fig. 2c).

In vitro Diagnosis of Wegener's Disease with Natural and Recombinant Pr3

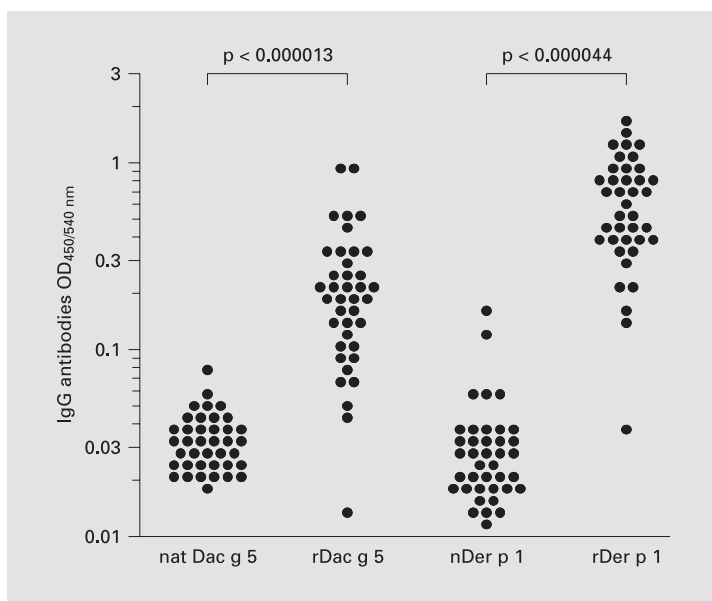
For diagnosing Wegener's disease, IgG autoantibodies to Pr3 were measured by sandwich ELISA. For patients with established Wegener's disease, IgG titers against natural Pr3 (nPr3) and rPr3 correlated closely (not shown). However, for healthy controls who were negative in the ELISA with nPr3, we frequently obtained high values in ELISAs with rPr3, as shown for 3 controls in figure 3a. This binding of IgG to rPr3 was inhibited by up to 82% (62–11 AU/ml) by yeast mannan (250 µg/ml) and by up to 99% (62–10 AU/ml) with high concentrations of free mannose (50 mM) (fig. 3a). Partial inhibition of up to 43% (0.512–0.297 OD) was observed with α -(1,3), α -(1,6)-mannotriose- α -methyl-glycoside (fig. 3b). Partial or no inhibition of IgG binding was observed for patients with Wegener's disease (<43% with mannan and <31% with mannose).

Inclusion of mannose at 200 mmol/l in the ELISA incubation buffer dramatically lowered the binding of sera from healthy controls to rPr3. We adopted a cut-off value of 10 U/ml for the inclusions (data not shown); this was based on the mean of 99 control donors $\pm$ 2 SD, which gave a value of 5.7 U/ml. We considered a cut-off value of 10 U/ml to be on the safe side.

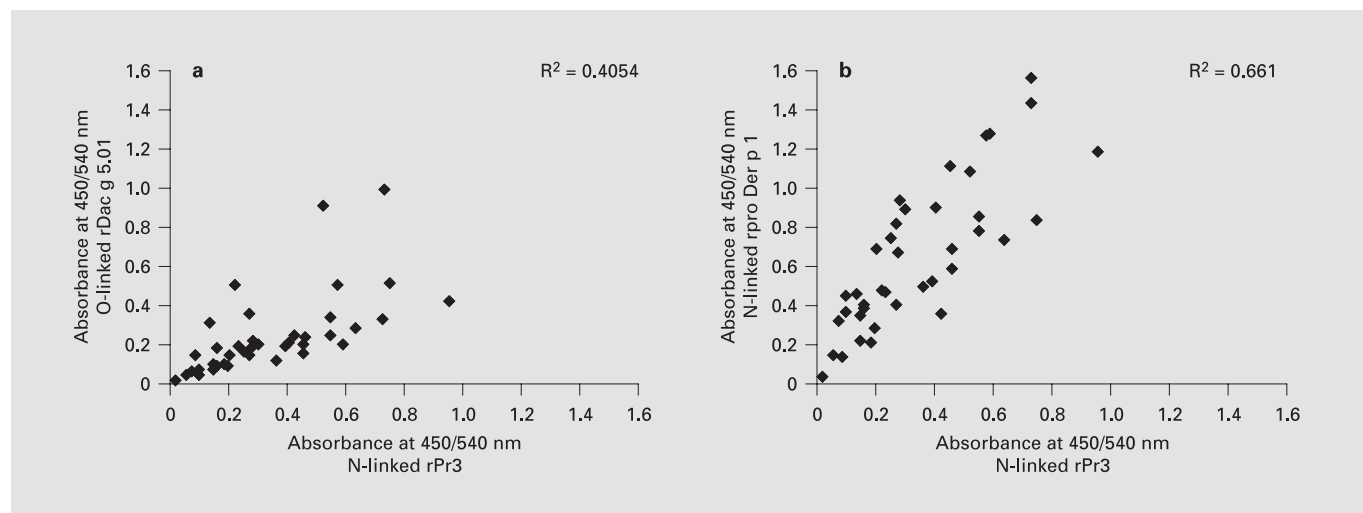
To further confirm that mannose-containing glycans on *P. pastoris*-derived glycoproteins can cause false-positive ELISA results, we measured IgG antibodies against O-glycosylated rDac g 5 and N-glycosylated rproDer p 1, and compared these results to those obtained with their natural homologues in the sera of 39 healthy volunteers. The mean OD values were 0.033 (nDac g 5), 0.246 (rDac g 5), 0.033 (nDer p 1) and 0.661 (rDer p 1), respectively. These results show a significant difference in response between natural and yeast-derived proteins ($p < 0.000013$ and $p < 0.000044$) (fig. 4). No cut-off for this ELISA was

Fig. 4. Detection of IgG antibodies against yeast-derived glycans in healthy control sera. The IgG responses of 39 healthy controls towards natural non-glycosylated Dac g 5, its yeast-derived O-linked glycosylated counterpart (rDac g 5), nDerp1 and yeast-derived rproDer p 1 (rDer p 1) are shown, respectively.

Fig. 5. Comparison of IgG responses towards O- and N-linked glycans. **a** Correlation between N-linked glycosylated rPr3 (x-axis) and O-linked glycosylated rDac g 5 (y-axis). **b** Correlation between N-linked glycosylated rPr3 (x-axis) and N-linked glycosylated rproDer p 1 (y-axis).



4



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determined, as IgG against natural Dac g 5 or Der p 1 is expected to be found in random blood donor samples.

IgG titers against rPr3 and the 2 unrelated allergens showed a significant correlation when analyzed by linear regression and Spearman correlation (rPr3 versus rDac g 5, $r_s = 0.787$; rPr3 vs. rDer p 1, $r_s = 0.838$) (fig. 5a, b). The highest responses were found with rproDer p 1, containing 2 consensus N-linked glycosylation sites.

In vitro Diagnosis of Allergy by RAST with Natural and Recombinant Allergens

To evaluate the potential interference of yeast glycans with the specificity of RAST, sera of ABPA patients with

A. fumigatus-specific IgE antibodies ($n = 9$) were tested in a RAST with nDac g 5 and rDac g 5 and/or nDer p 1 and rproDer p 1. Two sera (22%) had significantly higher IgE titers to rDac g 5 than to nDac g 5: 2.1 versus 0.6 and 1.8 versus 0.5 IU/ml, respectively. The other ABPA patients did not show a difference in response towards recombinant and natural Dac g 5: 0.04–0.04, 0.3–0.3, 0.09–0.05, 0.05–0.1, 0.16–0.13, 0.3–0.2 and 0.4–0.4 IU/ml, respectively. These results suggest that IgE responses to yeast glycans can give rise to false-positive in vitro tests in some patients with mould- and/or yeast-reactive IgE antibodies when yeast recombinant proteins are used.

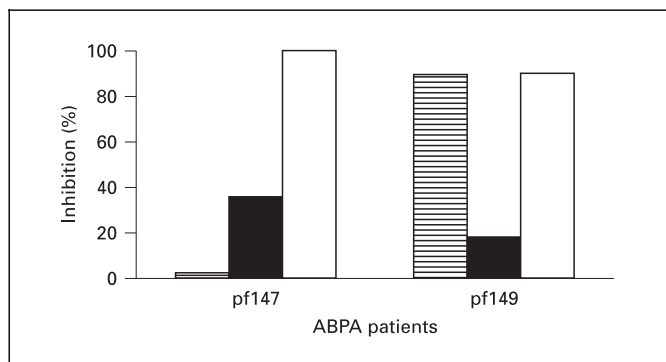


Fig. 6. Specificity of in vitro allergy diagnosis by RAST. The interference of yeast glycans in RAST was demonstrated in 2 out of 9 sera. Binding of IgE to rDac g 5 coupled to Sepharose could be inhibited by *Aspergillus* extract (▨), nDac g 5 (■) and rDac g 5 (□), respectively.

IgE binding to rDac g 5 was only partially inhibited by nDac g 5 (<35%), but almost completely inhibited by *A. fumigatus* extract (>88%) in one serum. This suggests that mould glycans are cross-reactive with the glycans on the *P. pastoris*-derived grass pollen allergen (fig. 6).

Discussion

Glycosylation of proteins expressed in the yeast *P. pastoris* has been studied in detail by various groups. Both N- and O-linked glycans are dominated by mannose residues [3, 7–10, 26, 27]. We have demonstrated that expression of the major grass pollen allergen Dac g 5 in *P. pastoris* results in substitution with mannose containing O-glycans not found in the natural allergen. The exact structure has not yet been elucidated, but it is most likely in close agreement with earlier reports showing that *P. pastoris* substitutes serines and threonines with 1–4 mannose residues [3, 27–30]. The N-terminal peptide of rDac g 5 was shown to contain probably 4–7 mannose residues. This suggests that 2 or more of the 4 threonines found in the first 36 amino acids of rDac g 5 are O-glycosylated. On the basis of earlier mass-spectrometric comparison of rDac g 5 and nDac g 5, the former was found to be 2,500 Da larger than the natural allergen [18]. This suggests that the recombinant allergen harbors more O-glycans than the ones accounted for in the N-terminal peptide (7 mannose residues: 1,134 Da). Mannose phosphate diester groups have also been reported for *P. pastoris*-derived glycoproteins [7–9]. Whether phosphorylation also plays a role for O-linked glycosylated rDac g 5 is still under investigation.

The proteins with N-glycosylation sites that we studied (the autoantigen Pr3 and the major house dust mite allergen Der p 1) are both glycosylated by *P. pastoris*, probably with high-mannose glycans. In this study, we did not analyze the structure of N-glycans on both proteins, but clear recognition by ConA supports the presence of high-mannose N-glycans. When rPr3 was used in an ELISA for diagnosis of Wegener's autoimmune disease, around two-thirds of the healthy controls were falsely positive. This was not the case when neutrophil lysates were used as a source for nPr3. Several observations support IgG binding to mannose-rich yeast glycans as the explanation for the low specificity of the rPr3-based ELISA, for example, the strong correlation between IgG responses against rPr3 and 2 structurally completely unrelated allergen molecules, i.e. rDac g 5 and rproDer p 1. The only common structural feature between the 3 molecules is that they all hold mannose-containing glycans. The correlation found between IgG responses to O-glycans on rDac g 5 and N-glycans on rPr3 suggests that IgG antibodies against yeast glycans are cross-reactive with the 2 types of glycan structures. The mannose-dominated nature of the IgG epitope causing false-positive ELISA results was further supported by our observation that yeast mannan and even free mannose residues almost completely inhibit the binding to rPr3 in healthy individuals.

The high prevalence of IgG antibodies directed to yeast glycans observed in healthy subjects can most likely be explained by very regular environmental and dietary exposure to yeast and mould carbohydrate structures. In sera of a specific patient group, 22% showed false-positive IgE antibody responses to yeast glycans. Several other investigators have also reported a significant role for mannan-specific IgE antibodies in mould- and yeast-sensitized patients [31, 32]. In our study, 2 sera out of 9 from patients with ABPA demonstrated IgE antibodies against mould glycans that cross-reacted with the glycans on rDac g 5. Further serum screening will have to clarify whether IgE antibodies against mannose-rich carbohydrate structures are relevant for other mould- or yeast-allergic patients, thereby decreasing the specificity of RAST as a diagnostic tool for type I allergies.

In summary, our study has revealed that expression of proteins in *P. pastoris* results in their substitution with N- and O-glycans that are recognized by IgG antibodies in the majority of sera of healthy controls. This seriously decreases the specificity of diagnostic tests with such recombinant proteins. Glycosylation of diagnostic reagents produced in *P. pastoris* should therefore be carefully evaluated, and if prevention of unwanted glycosylation

proves to be impossible, alternative expression systems should be considered. Several new strains of *E. coli* have become available that better facilitate disulfide bridge formation and correct folding of recombinant proteins than the earlier strains. The choice between eukaryotic and prokaryotic expression systems depends on the characteristics of each individual protein.

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References

- Ballou C: Structure and biosynthesis of the mannan component of the yeast cell envelope. *Adv Microb Physiol* 1976;14:93–158.
- Hayette MP, Strecker G, Faille C, Dive D, Camus D, Mackenzie DW, et al: Presence of human antibodies reacting with *Candida albicans* O-linked oligomannosides revealed by using an enzyme-linked immunosorbent assay and neoglycolipids. *J Clin Microbiol* 1992;30:411–417.
- Letourneur O, Gervasi G, Gaia S, Pages J, Watelet B, Jolivet M: Characterization of *Toxoplasma gondii* surface antigen 1 (SAG1) secreted from *Pichia pastoris*: Evidence of hyper O-glycosylation. *Biotechnol Appl Biochem* 2001;33:35–45.
- Podzorski RP, Gray GR, Nelson RD: Different effects of native *Candida albicans* mannan and mannan-derived oligosaccharides on antigen-stimulated lymphoproliferation in vitro. *J Immunol* 1990;144:707–716.
- Kawai T, Suzuki Y, Eda S, Ohtani K, Kase T, Sakamoto T, et al: Molecular and biological characterization of rabbit mannan-binding protein. *Glycobiology* 1998;8:237–244.
- Herscovics A, Orlean P: Glycoprotein biosynthesis in yeast. *FASEB J* 1993;7:540–550.
- Hirose M, Kameyama S, Ohi H: Characterization of N-linked oligosaccharides attached to recombinant human antithrombin expressed in the yeast *Pichia pastoris*. *Yeast* 2002;19:1191–1202.
- Varki A, Kornfeld S: Structural studies of phosphorylated high mannose-type oligosaccharides. *J Biol Chem* 1980;255:10847–10858.
- Ballou L, Hernandez LM, Alvarado E, Ballou CE: Revision of the oligosaccharide structures of yeast carboxypeptidase Y. *Proc Natl Acad Sci USA* 1990;87:3368–3372.
- Gemmill TR, Trimble RB: Overview of N- and O-linked oligosaccharide structures found in various yeast species. *Biochim Biophys Acta* 1999;1426:227–237.
- Ballou CE: A study of the immunochemistry of three yeast mannans. *J Biol Chem* 1970;245:1197–1203.
- Spencer JF, Gorin PA: Mannose-containing polysaccharides of yeasts. *Biotechnol Bioeng* 1973;15:1–12.
- Specks U: What you should know about PR3-ANCA. Conformational requirements of proteinase 3 (PR3) for enzymatic activity and recognition by PR3-ANCA. *Arthritis Res* 2000;2:263–267.
- Chua KY, Stewart GA, Thomas WR, Simpson RJ, Dilworth RJ, Plozza TM, et al: Sequence analysis of cDNA coding for a major house dust mite allergen, Der p 1. Homology with cysteine proteases. *J Exp Med* 1988;167:175–182.
- Jacquet A, Haumont M, Massar M, Daminet V, Garcia L, Mazzu P, et al: Biochemical and immunological characterization of a recombinant precursor form of the house dust mite allergen Der p 1 produced by *Drosophila* cells. *Clin Exp Allergy* 2000;30:677–684.
- Chapman MD, Platts-Mills TA: Purification and characterization of the major allergen from *Dermatophagoides pteronyssinus*-antigen P1. *J Immunol* 1980;125:587–592.
- Van Oort E, de Heer PG, van Leeuwen WA, Derksen NI, Muller M, Huvenneers S, et al: Maturation of *Pichia pastoris*-derived recombinant pro-Der p 1 induced by deglycosylation and by the natural cysteine protease Der p 1 from house dust mite. *Eur J Biochem* 2002;269:671–679.
- Van Oort E, de Heer PG, Lerouge P, Faye L, Aalberse RC, van Ree R: Immunochemical characterization of two *Pichia pastoris*-derived recombinant group 5 *Dactylis glomerata* isoallergens. *Int Arch Allergy Immunol* 2001;126:196–205.
- Qin S, Tang H, Zhao LS, He F, Lin Y, Liu L, et al: Cloning of HBsAg-encoded genes in different vectors and their expression in eukaryotic cells. *World J Gastroenterol* 2003;9:1111–1113.
- Van Ree R, Clemens JG, Aalbers M, Stapel SO, Aalberse RC: Characterization with monoclonal and polyclonal antibodies of a new major allergen from grass pollen in the group I molecular weight range. *J Allergy Clin Immunol* 1989;83:144–151.
- Van der Zee JS, van Swieten P, Jansen HM, Aalberse RC: Skin tests and histamine release with P1-depleted *Dermatophagoides pteronyssinus* body extracts and purified P1. *J Allergy Clin Immunol* 1988;81:884–896.
- Goldschmeding R, van der Schoot CE, ten Bokkel HD, Hack CE, van den Ende ME, Kaltenberg CG, et al: Wegener's granulomatosis autoantibodies identify a novel diisopropyl-fluorophosphate-binding protein in the lysosomes of normal human neutrophils. *J Clin Invest* 1989;84:1577–1587.
- Faye L, Chrispeels MJ: Characterization of N-linked oligosaccharides by affino blotting with concanavalin A-peroxidase and treatment of the blots with glycosidases. *Anal Biochem* 1985;149:218–224.
- Aalberse RC, Koshte V, Clemens JG: Immuno-globulin E antibodies that crossreact with vegetable foods, pollen, and Hymenoptera venom. *J Allergy Clin Immunol* 1981;68:356–364.
- Schuurman J, Perdok GJ, Lourens TE, Parren PW, Chapman MD, Aalberse RC: Production of a mouse/human chimeric IgE monoclonal antibody to the house dust mite allergen Der p 2 and its use for the absolute quantification of allergen-specific IgE. *J Allergy Clin Immunol* 1997;99:545–550.
- Duman JG, Miele RG, Liang H, Grella DK, Sim KL, Castellino FJ, et al: O-Mannosylation of *Pichia pastoris* cellular and recombinant proteins. *Biotechnol Appl Biochem* 1998;28:39–45.
- Boraston AB, Sandercock LE, Warren RA, Kilburn DG: O-Glycosylation of a recombinant carbohydrate-binding module mutant secreted by *Pichia pastoris*. *J Mol Microbiol Biotechnol* 2003;5:29–36.
- Mochizuki S, Hamato N, Hirose M, Miyano K, Ohtani W, Kameyama S, et al: Expression and characterization of recombinant human antithrombin III in *Pichia pastoris*. *Protein Expr Purif* 2001;23:55–65.
- Wartmann T, Stephan UW, Bube I, Boer E, Melzer M, Manteuffel R, et al: Post-translational modifications of the AFET3 gene product: A component of the iron transport system in budding cells and mycelia of the yeast *Arxula adenivorans*. *Yeast* 2002;19:849–862.
- Van den SP, Rudd PM, Proost P, Martens E, Paemen L, Kuster B, et al: Oligosaccharides of recombinant mouse gelatinase B variants. *Biochim Biophys Acta* 1998;1425:587–598.
- Nermes M, Savolainen J, Kortekangas-Savolainen O: Nitrocellulose-RAST analysis of allergenic cross-reactivity of *Candida albicans* and *Saccharomyces cerevisiae* mannans. *Int Arch Allergy Immunol* 1995;106:118–123.
- Kortekangas-Savolainen O, Kalimo K, Lam-mintausta K, Savolainen J: IgE-binding components of baker's yeast (*Saccharomyces cerevisiae*) recognized by immunoblotting analysis. Simultaneous IgE binding to mannan and 46–48 kD allergens of *Saccharomyces cerevisiae* and *Candida albicans*. *Clin Exp Allergy* 1993;23:179–184.