

Immunochemical Characterization of Two *Pichia pastoris*-Derived Recombinant Group 5 *Dactylis glomerata* Isoallergens

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Key Words

Grass pollen · *Dactylis glomerata* · Allergy · Isoallergens · Recombinant proteins · Allergenicity · *Pichia pastoris*

Abstract

Background: Grass pollen of the Poaceae grasses are known to be highly allergenic. Major allergens from the species *Lolium*, *Phleum*, *Poa* and *Holcus* have been cloned and expressed as recombinant proteins, but of the important species *Dactylis glomerata* no recombinants are available. **Methods:** Dac g 5 was cloned by PCR on the basis of homology with Lol p 5 and expressed in *Pichia pastoris*. Recombinant Dac g 5 (rDac g 5) was affinity purified and compared to natural Dac g 5 (nDac g 5) by immunoblot, radioallergosorbent test (RAST), RAST inhibition, basophil histamine release assay (HRA), competitive radioimmunoassay (RIA) and sandwich enzyme-linked immunosorbent assay (ELISA). In addition, N-terminal sequencing, concanavalin A (Con A) binding, circular dichroism spectrum measurements and matrix-assisted laser desorption ionization-time of flight mass-spectrometric analysis were performed. **Results:** Clones were obtained that coded for pro-Dac g 5 and two mature isoforms of Dac g 5; the deduced amino acid sequences of both isoforms differed by 4 amino acids. Both mature

isoforms were expressed in *Pichia* at a concentration of ~ 15 mg/l. SDS-PAGE analysis showed that rDac g 5 had an apparent M_r approximately 10 kD above nDac g 5. By mass spectrometry this difference was shown to be around 2.5 kD. Positive Con A staining suggested (O-linked) glycosylation as an explanation for this increase in M_r . Whereas both purified recombinants showed a tendency to dimerize, purified nDac g 5 contained a 12-kD peptide not observed for rDac g 5. RAST, RAST inhibition and HRA showed that the IgE reactivity of rDac g 5 was similar to that of nDac g 5. A small subgroup, however, clearly demonstrated decreased IgE reactivity to rDac g 5.02. Differences in immune reactivity of both isoforms were confirmed by monoclonal antibody (mAb)-based sandwich ELISA. **Conclusions:** Dac g 5 was successfully cloned and expressed in *P. pastoris*. Minor differences in primary structure between isoforms influence their immune reactivity.

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Introduction

In temperate latitudes, pollen from Poaceae grasses like *Dactylis glomerata* (orchard grass), *Lolium perenne* (rye grass), *Poa pratensis* (Kentucky blue grass) and *Phleum pratense* (Timothy grass) are a major cause of sea-

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sonal atopic allergy. To date grass pollen allergens are divided into 11 groups [1, 2], of which group 1 and group 5 are identified as the most important allergens [3–6].

Several group 5 allergens have already been cloned and/or immunologically characterized [7–14]. Group 5 allergens are reported to be nonglycosylated, highly homologous proteins with a molecular mass (M_r) ranging from 25 to 33 kD [15–17]. Purified natural group 5 allergens were shown to contain significant amounts of dimers and of a 12-kD cleavage peptide [own unpubl. obs., 6, 18]. The exact nature of the low-molecular-mass (LMM) peptide has so far not been clarified, although Bufe et al. [19] reported spontaneous occurrence of a C-terminal peptide of similar size in *Escherichia coli*-derived recombinant Phl p 5. This C-terminal peptide of Phl p 5 was crystallized and, on the basis of homology and functional assays, proposed to have a role as RNase [18]. These observations have so far not been confirmed by other groups. Lol p 5 is further shown to be located in the starch granules of pollen with a possible role in starch mobilization and plant germination [7]. Therefore, the structure, function and allergenicity relationships of group 5 allergens are still under discussion [19, 20].

In contrast to the group 1 and group 5 allergens of *Lolium* (Lol p), *Poa* (Poa p) and *Phleum* (Phl p), the allergens of *Dactylis* (Dac g) are (to the best of our knowledge) still unavailable as recombinant proteins. It has been proposed that recombinant allergens are suitable as replacements for natural allergens in the diagnosis of allergy, (e.g. skin prick testing, radioallergosorbent test (RAST) and as an alternative for crude extracts currently in use for immunotherapy [21–24]. Pollen allergens have so far largely been expressed in *E. coli*. Some grass group 1 allergens (Phl p 1, Cyn d 1 and Lol p 1) and the major olive pollen allergen Ole e 1 [25] were also expressed in the methylotrophic yeast *P. pastoris*. As an eukaryote, *Pichia*

has the advantages of protein processing, protein folding and posttranslational modifications. This advantage becomes evident from the IgE reactivity displayed by the group 1 allergens expressed in *E. coli* compared to their expression in *Pichia* [26–30]. Also for *E. coli*-derived recombinant group 5 allergen a lower IgE-binding capacity was shown compared with its natural counterpart [22]. In addition *Pichia* enables secretion of recombinant proteins in the culture medium at high expression levels. We describe the expression and immunochemical characterization of two isoforms of group 5 grass pollen allergens of *D. glomerata* in *P. pastoris*.

Materials and Methods

Cloning of Dac g 5

Total RNA was extracted from *D. glomerata* (Dac g) grass pollen (ALK, Hørsholm, Denmark) using TRIzol (Life Technologies, Gaithersburg, Md., USA) following the manufacturer's instructions. cDNA was generated with Superscript RNaseH Reverse Transcriptase (Gibco BRL) following the manufacturer's instructions and PCR was performed with primers (Gibco BRL) designed on the basis of Lol p 5 homologies (table 1). PCR products were cloned into the pGem-T vector system (Promega Benelux, Leiden, The Netherlands) for sequencing. DNA sequences were determined by automated sequencing (Applied Biosystems, USA) using the DYEnamic™ ET terminator cycle sequencing premix kit (Amersham Pharmacia Biotech, Inc., USA) according to the manufacturer's instructions. From the sequence, two *D. glomerata*-specific internal primers were generated (table 1) for 5'RACE and 3'RACE (Life Technologies). The RACE fragments were obtained according to the supplier's protocol and subsequently cloned into pGem-T vector (Promega) and sequenced. The complete 5' and 3' ends of Dac g 5 were obtained. Primers were constructed to obtain both the mature and the proenzyme of Dac g 5. The signal sequence or pro-sequence of Dac g 5 starts with ATGGCGGTCCAGAAGTACACC (table 1). After sequencing, inserts coding for two isoforms of mature Dac g 5 were selected. This selection was based on confirmation of the sequences in two or more clones obtained from independent PCRs.

Table 1. Primers used for cloning and sequencing of both group 5 recombinant proteins of *D. glomerata*

Primer name	Primer sequence
Group 5 homologous (5' primer)	5'GCTGACGCCGGCTACACC3'
Group 5 homologous (3' primer)	5'TCAGACTTTGTAGCCACC3'
5' RACE primer	5'CTTGGTCAGCGCGGCCTCAAA3'
3' RACE primer	5'GTCTTTGAGGCCGCGCTGACC3'
pPICZaA cloning primer (5' mature)	5'AAGCTCGAGAAAAGAGCCGACGCCGGCTACACC3'
pPiCZaA cloning primer (5' pro-sequence)	5'GGGTCTAGAATGGCGGTCCAGAAGTACACC3'
pPICZaA cloning primer (C-terminal primer)	5'GGGGAGCTCTCAGACTTTGTAGCCACCGGC3'
Sequence primers used in pGEM-T vector	pUC/M13R and pUC/M13F or T7, SP6
Sequence primers used in pPICZaA	5'AOX, 3'AOX and α -factor primer

DNA to protein translation was performed by using the medkem translation program on the Internet (www.medkem.gu.se/edu/translation.html). Amino acid alignments were performed in clustalw analysis, protein sequences of group 5 allergens were incorporated from the Genbank database. The DNA and amino acid sequences of a pro-allergen (pro-Dac g 5) and two mature Dac g 5 recombinant proteins (rDac g 5.01 and rDac g 5.02) were submitted to the Genbank database with accession numbers: AF325267, AF325265 and AF325266, respectively.

Expression in P. pastoris

cDNA's coding for Dac g 5.01 and Dac g 5.02 were cloned into the pPICZαA vector in frame with the α-factor prepro-secretion signal sequence from *Saccharomyces cerevisiae*. By using this vector (pPICZαA), the recombinant proteins will be secreted into the medium. Transformation into *P. pastoris* strain SMD1168H (PEP4 mutant, deficient in protease A, HIS4+) was carried out as described by the manufacturer (Invitrogen, San Diego, Calif., USA). Positive clones were selected from YPD plates (Yeast extract Peptone Dextrose Medium) containing Zeocine (100 µg/ml) as a selection marker. Selected clones were inoculated in YPD with Zeocine and grown overnight at 29°C. Subsequently, the cells were transferred to BMGY (Buffered Glycerol-complex Medium) for 24 h after which the cells were centrifuged (glycerol inhibits expression) and transferred to expression medium (buffered methanol-complex medium, BMMY, pH 6.0) at OD_{600nm} 10. After 5 days, the supernatant was harvested.

Purification of (Recombinant) Allergens

rDac g 5 was purified from culture supernatant by affinity purification with a group 5-specific monoclonal antibody (4B1) as described elsewhere [31]. Purity was assessed by SDS-PAGE/silver staining. The natural allergen was purified from a Dac g extract by the same method. Protein concentrations were determined, using the BCA method as described by the manufacturer (Pierce, Rockford, Ill., USA).

SDS-PAGE and Immunoblotting

Proteins were separated by SDS-PAGE (4–12%) (Novex, San Diego, Calif., USA), as described by the manufacturer and silver stained according to the ExcelGel protocol (Amersham Pharmacia Biotech, Uppsala, Sweden). Western blotting was performed by transferring the proteins onto nitrocellulose membrane as described by the manufacturer (Novex). Subsequently, the blots were blocked with PBS-A (PBS, 1% BSA) and incubated with group 5 specific monoclonal or polyclonal antibodies. After washing, the blots were incubated overnight with ¹²⁵I-labeled goat anti-mouse IgG, rat anti-mouse kappa light chain or sheep anti-rabbit IgG antibodies (CLB, Amsterdam, The Netherlands) and exposed to an autoradiographic film (Eastman Kodak Company, Rochester, N.Y., USA).

RAST and RAST Inhibition

A radioallergosorbent test (RAST) was performed as described previously [32]. Briefly, both natural and recombinant proteins were coupled to CNBr-activated Sepharose 4B (100 µg/100 mg Sepharose; Amersham Pharmacia Biotech). The Sepharose was suspended at a concentration of 2 mg/ml in PBS-AT (phosphate-buffered saline/0.3% BSA/0.1% Tween), of which 250 µl were incubated with 50 µl of human serum. After incubation overnight, unbound material was washed away, and 50 µl of ¹²⁵I-labeled sheep anti-human IgE (CLB)

were added. After incubation overnight and washing, radioactivity was measured on a gamma-counter. The results were expressed as IU/ml which were calculated from a standard curve of serial dilutions of a human/mouse chimeric IgE antibody directed to Der p 2 and Sepharose-coupled rDer p 2 [33]. A result greater than 0.30 IU/ml was regarded as positive.

RAST inhibition was performed by preincubation of a serum pool (n > 100) with 50 µl rDac g 5.01, rDac g 5.02 and nDac g 5 (1 ng/ml–10 µg/ml) before adding Sepharose-coupled nDac g 5. The samples were further treated as described for the RAST.

Radiolabeling

Purified nDac g 5 (25 µg) was radiolabeled with ¹²⁵I using the chloramine-T method. Radiolabeled allergen and free iodine were separated by size exclusion chromatography. Three different ¹²⁵I-nDac g 5 peaks were obtained: dimer, monomer and 12-kD peptide.

Competitive Radioimmunoassay

In a competitive radioimmunoassay (RIA) [22, 34], 50 µl of a rabbit anti-group 5 antibody (1 : 3,000) [31] were preincubated for 2 h at room temperature with 50 µl of serial dilutions of the inhibitor (rDac g 5, nDac g 5, whole pollen extract and *Pichia* culture supernatants), before addition of 250 µl Protein A Sepharose (2 mg/ml), after which 50 µl of ¹²⁵I-labeled nDac g 5 (monomer, dimer or 12-kD peptide) were added and incubated overnight at room temperature. For the uninhibited value, polyclonal antibody was preincubated with PBS-AT instead of inhibiting allergen.

Grass Pollen Group 5 Sandwich ELISA

A grass pollen group 5 monoclonal-antibody (mAb)-based sandwich ELISA was developed with capturing antibody 14C2 (anti-Phl p 5), 2 µg/ml in coating buffer (50 mM sodium carbonate, pH 9.6) and detecting antibody 13D9 (anti-Phl p 5), 150 ng/ml in blocking buffer (PBS, 1% BSA, 0.05% Tween-20) [manuscript in preparation]. Dilutions of the samples were made in blocking buffer and as a reference a dilution of total *D. glomerata* extract was used (0.156–20 µg/ml (total protein) in twofold dilutions). Both recombinants and purified natural Dac g 5 were tested in this ELISA in a range from 1.95 to 500 ng/ml in a twofold dilution. After each step, the plates were washed with PBS, 0.05% Tween-20. All incubations were performed at 37°C for 1 h, with the exception of Streptavidin-horseradish peroxidase conjugate, which was incubated for 30 min (Amersham Life Science). Color development with 2,2'-azinobis (3-ethylbenzthiazolinesulfonic acid) in substrate buffer (sodium citrate, pH 4.0) was performed in the dark at room temperature for 30 min. The reaction was stopped by adding 2% oxalic acid (Sigma) after which the absorbance was measured at 414 nm.

In vitro Histamine Release Assays

White blood cells were isolated from blood of a nonallergic donor by Percoll centrifugation and stripped from IgE by lactic acid treatment as described elsewhere [35, 36]. Subsequently, cells were resensitized with patients' sera (n = 6) testing positive (RAST) for group 5 allergen. Histamine release was performed with purified natural and recombinant Dac g 5 (1 ng/ml–1 µg/ml). Liberated histamine was measured essentially by the fluorometric method described by Siraganian [37]. The protocol was approved by the medical ethical committee (MEC) of the Amsterdam Medical Center under project number: MEC97/030.

Sugar Analysis by Concanavalin A Binding

Both recombinants and natural Dac g 5 were electroblotted onto nitrocellulose membrane followed by an overnight incubation in TBS/Tween 0.1%. Subsequently, the blot is incubated with concanavalin A (Con A; 25 µg/ml) in TBS/Tween 0.1%, 1 mM MgCl₂ and 1 mM CaCl₂ for 90 min. After washing with TBS/Tween 0.1%, 1 mM MgCl₂, 1 mM CaCl₂ the membrane was incubated with horseradish peroxidase for 60 min (50 µg/ml) (Sigma, St. Louis, Mo., USA) [38]. The bands were visualized with 1 tablet (10 mg) of diaminobenzidine-tetrahydrochloride (Kem-En-Tec, Copenhagen, Denmark) in distilled water. The reaction was started with 40 µl of 30% H₂O₂.

Mild periodate oxidation was performed by incubating the blot in a 100 mM sodium acetate buffer containing 100 mM sodium periodate for 1 h at room temperature in the dark. After washing, the blot was incubated in PBS containing 50 mM sodium borohydride for 30 min. The blot was rinsed and affinity detection by Con A was performed as described above.

Circular Dichroism

In circular dichroism (CD) experiments, ellipticity measurements were performed either with nDac g 5, rDac g 5.01 and 5.02 (2 mg/ml) dissolved in water. Spectra were recorded on a CD6 dichrograph (Jobin Yvon) in 1 or 0.1 mm path length quartz cells (Hellma). The instrument was calibrated using (+)-10-camphorsulfonic acid.

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra of the proteins were recorded on a Micromass ToF spec E mass spectrometer (Manchester, UK). This instrument was operated to an accelerating voltage of 25 kV in linear mode and a pressure of approximately 10⁻⁷ mbar in the source and 10⁻⁶ mbar in the analyzer. The nitrogen laser wavelength was set at 337 nm and a pulse width of 4 ns. The MALDI-TOF mass spectra were performed in positive ion mode using a postacceleration detector of 15 keV. They were smoothed once and externally calibrated with apomyoglobin (16,952 kD) and cytochrome c (12,361 kD). The laser shots were summed for each mass spectrum in order to obtain an acceptable signal-to-noise ratio. Two microliters of the sample at a concentration around 10 pmol/µl were dissolved in the same volume of matrix solution containing 2 mg of sinapinic acid diluted in acetonitrile/water-TFA 0.1% (40/60 v/v). The sample-matrix mixture was homogenized and 1 µl was deposited onto the target and allowed to dry.

N-Terminal Sequencing

Both recombinant isoallergens were separated by SDS-PAGE and electroblotted on polyvinylidene difluoride membrane. Blots were stained with Coomassie R-250 in 50% methanol. The bands corresponding to rDac g 5.01 and 5.02 were excised and sequenced on a Perkin Elmer/Applied Biosystems 476A gas phase sequencer.

Sera

From sera submitted for routine in vitro allergy diagnostics, a panel of 59 sera was selected based on specific IgE antibodies directed to grass pollen (>0.30 IU/ml). Of this panel, 17 sera [negative controls (data not shown)] did not possess IgE antibodies specific for group 5 (<0.3 IU/ml). A pool (n >100) of grass-pollen-positive sera (>6.5 IU/ml) was used for RAST inhibition.

Statistical Analysis

The RAST results for nDac g 5 and both recombinant isoforms were analyzed by Spearman rank correlation. Responses in sandwich ELISA were compared by parallel-line analyses.

Results

Sequence Analyses of Two Mature and One Proallergen of Dac g 5

Two mature cDNAs and one cDNA containing the pro-sequence were obtained by performing two independent PCRs. Clones from both PCRs were sequenced for confirmation of the correct sequences. The sequences were each confirmed in at least two independent clones and submitted to the Genbank database with accession numbers: AF325265 (rDac g 5.01), AF325266 (rDac g 5.02) and AF325267 (proallergen).

The deduced protein sequences of rDac g 5.01 and rDac g 5.02 (265 aa) differed at 4 positions: 15 (T/A), 26 (T/K), 92 (T/A) and 240 (V/A). When compared with sequences of Phl p 5.0107 (AF061068), Phl p 5a [39], Hol l 5 [14], Phl p 5b [18] and Lol p 5c [40] from the Genbank database; the highest homology was observed with Lol p 5c (77%) and Phl p 5b (76%). Both share a single cysteine with our rDac g 5, not found in the other published group 5 allergens.

Expression of Two Mature Isoallergens of Dac g 5 in the Yeast P. pastoris

For expression of both mature recombinant isoforms of Dac g 5 the SMD1168H strain of *P. pastoris* was used. This resulted in an expression level of 16.7 µg/ml (rDac g 5.01) and 14.7 µg/ml (rDac g 5.02) in a competitive RIA. The purification of recombinant protein with a group 5 monoclonal antibody (4B1) gave a final yield of 30% of the starting material.

Both isoallergens and natural protein were separated on SDS-PAGE (4–12%) (Novex), silver stained (Amersham Pharmacia Biotech) and blotted on nitrocellulose membrane as is shown in figure 1a, b, d. Both recombinant isoforms migrated as a doublet at a higher apparent M_r (32/38 kD) than their natural counterpart (26/28 kD). It was further shown that a 12-kD fragment, which was recognized by mAb 4B1, was present in the purified nDac g 5 but not detected in purified rDac g 5. On the other hand, dimerization of Dac g 5 was mainly observed for the recombinant allergens, even under reducing conditions.

Both recombinants and natural Dac g 5 were subjected to binding to Con A on nitrocellulose membrane. As is shown in figure 1c, the lectin bound to both recombi-

Fig. 1. **a** SDS-PAGE silver-stained gel. rDac g 5.02 (lane 1), rDac g 5.01 (lane 2), nDac g 5 (lane 3) and the 10-kD ladder (lane 4). **b** Immunoblot with an mAb (4B1). rDac g 5.02 (lane 1), rDac g 5.01 (lane 2) and nDac g 5 (lane 3). This mAb also recognized the 12-kD cleavage peptide or splice variant present in nDac g 5. **c** Con A blot. rDac g 5.01 (lane 1), rDac g 5.02 (lane 2) and nDac g 5 (lane 3) were blotted on nitrocellulose membrane and probed with Con A. Both recombinants show binding to the lectin; nDac g 5 did not. **d** Immunoblot with group 5-specific mAb (4B1). nDac g 5 (lane 1). Concentration of natural Dac g 5 with Amicon 1-kD filter instead of a 10-kD filter improved the yield of the 12-kD peptide.

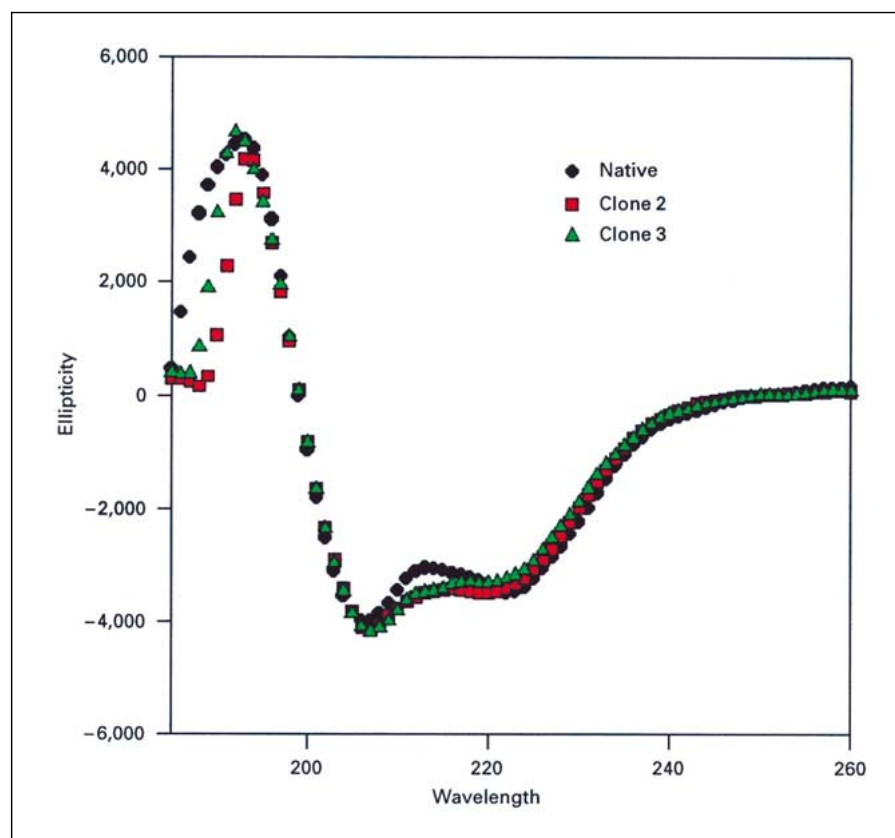
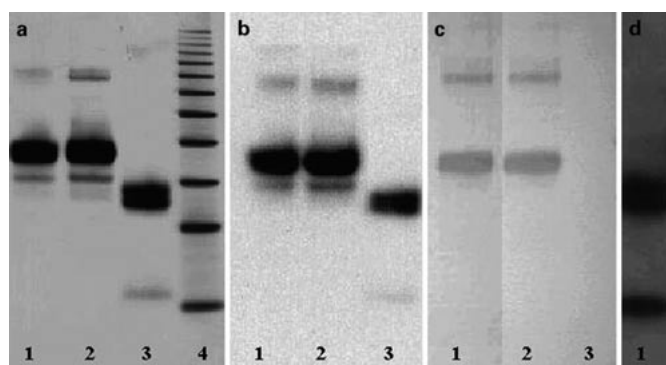


Fig. 2. CD spectra were measured for natural Dac g 5, rDac g 5.01 (clone 2) and rDac g 5.02 (clone 3).

nants, but not to the natural protein. After sodium periodate treatment of the blot, the binding of Con A was lost (data not shown). Together these observations suggest that the recombinant allergens contain O-linked glycans since Dac g 5 lacks putative N-glycosylation sites.

N-Terminal Sequencing, CD Spectra and MALDI-TOF Analysis

N-terminal sequencing was performed on both recombinants to investigate whether inefficient cleavage of the

yeast secretion peptide could explain their higher apparent M_r observed on SDS-PAGE. It was shown that the first 10 amino acids were identical to the N-terminal amino acid sequence of natural Dac g 5 (ADAGYTPAAA) [16].

CD spectrum measurements revealed minor differences in the two-dimensional structure between both recombinants and their natural counterpart (fig. 2).

The M_r of rDac g 5.01 and rDac g 5.02 determined by MALDI-TOF were $28.6 \text{ kD} \pm 150 \text{ D}$ and $28.9 \text{ kD} \pm$

170 D, respectively, being approximately 2.5 kD above the theoretical mass calculated from their amino acid sequences. The mass of nDac g 5 was determined by MALDI-TOF at $24.3 \text{ kD} \pm 75 \text{ D}$.

IgE Reactivity (RAST, RAST-Inhibition and HRA)

Grass-pollen-allergic patients were tested by a RAST ($n = 59$) for specific IgE antibodies against nDac g 5 and both recombinant isoallergens (fig. 3). By Spearman rank correlation, IgE responses to nDac g 5 on the one hand and the IgE responses of both recombinants on the other hand, were shown to closely correlate: $R_s = 0.9528$, $P_s < 0.0001$ (rDac g 5.01 with nDac g 5), $R_s = 0.8714$, $P_s < 0.0001$ (rDac g 5.02 with nDac g 5) and $R_s = 0.9436$, $P_s < 0.0001$ (rDac g 5.02 with rDac g 5.01). Despite these close correlations six sera showed decreased recognition of rDac g 5.02 (4–22 times lower).

By RAST inhibition with a serum pool, no significant differences in inhibitory potencies were observed between the three reagents (data not shown).

By HRA, 6 patients were tested for their ability to induce histamine release with nDac g 5, rDac g 5.01 and rDac g 5.02. Both recombinants showed similar or slightly decreased biological activity as is shown in figure 4a–c with 3/6 patients as representatives. Stripped cells were used as a negative control and showed a histamine release of 2% (data not shown). To obtain a release of 30% (patient A), a concentration of respectively 4.0 ng/ml (nDac g 5), 2.0 ng/ml (rDac g 5.01) and 0.9 ng/ml (rDac g 5.02) had to be added. A release of 50% (patient B) respectively, 2.4 ng/ml (nDac g 5), 2.4 ng/ml (rDac g 5.01) and 1.3 ng/ml (rDac g 5.02). A release of 28% (patient C) needed respectively 0.70 ng/ml (nDac g 5), 1.5 ng/ml (rDac g 5.01) and 0.90 ng/ml (rDac g 5.02).

Major Allergen Tests (Competitive RIA, sandwich ELISA)

nDac g 5, rDac g 5.01 and rDac g 5.02 were compared using a competitive RIA with three different labeled nDac g 5 preparations (monomer, dimer and a 12-kD peptide). No significant differences were observed between both recombinant isoforms (fig. 5a–c). Binding of radiolabeled dimer was inhibited more efficiently by rDac g 5 than by nDac g 5 (fig. 5b). In contrast, there was a trend that nDac g 5 more potently inhibited the binding of the 12-kD peptide (fig. 5c). When whole Dac g extract was used as inhibitor, inhibition of binding of the dimer was approximately 100-fold less efficient than that of monomer and the 12-kD peptide (fig. 5d).

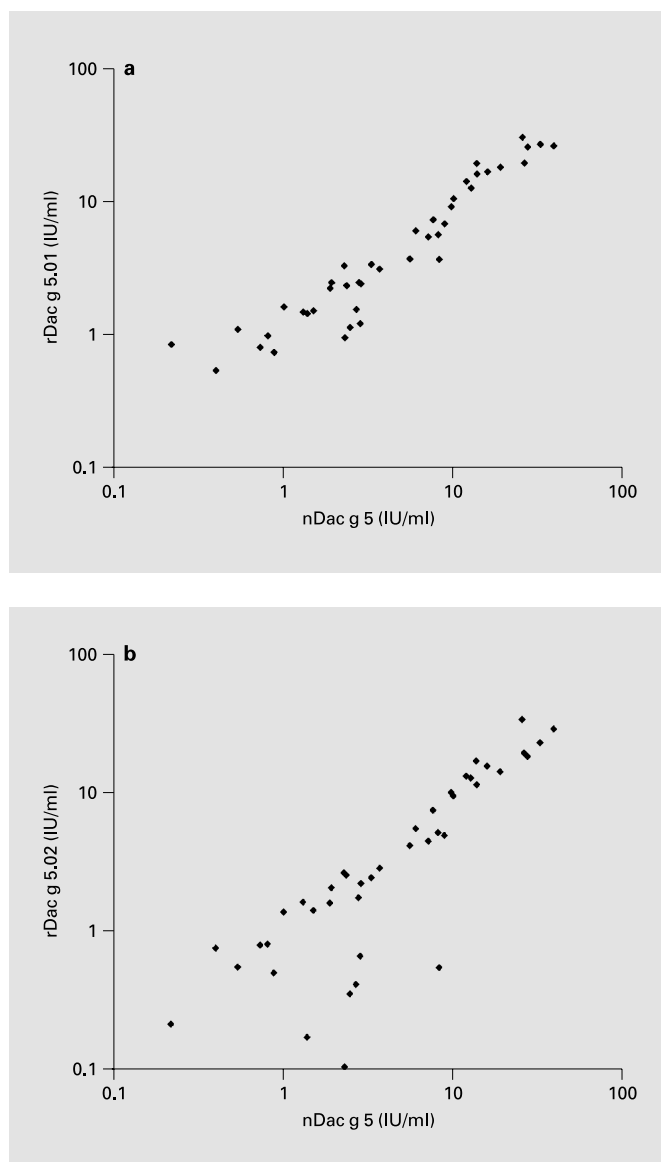


Fig. 3. RAST with 42 grass-pollen-positive patients. **a** IgE responses to nDac g 5 versus rDac g 5.01, expressed in IU/ml. **b** IgE responses to nDac g 5 versus rDac g 5.02, expressed in IU/ml. Decreased reactivity towards isoform 5.02 is observed for 6/42 sera.

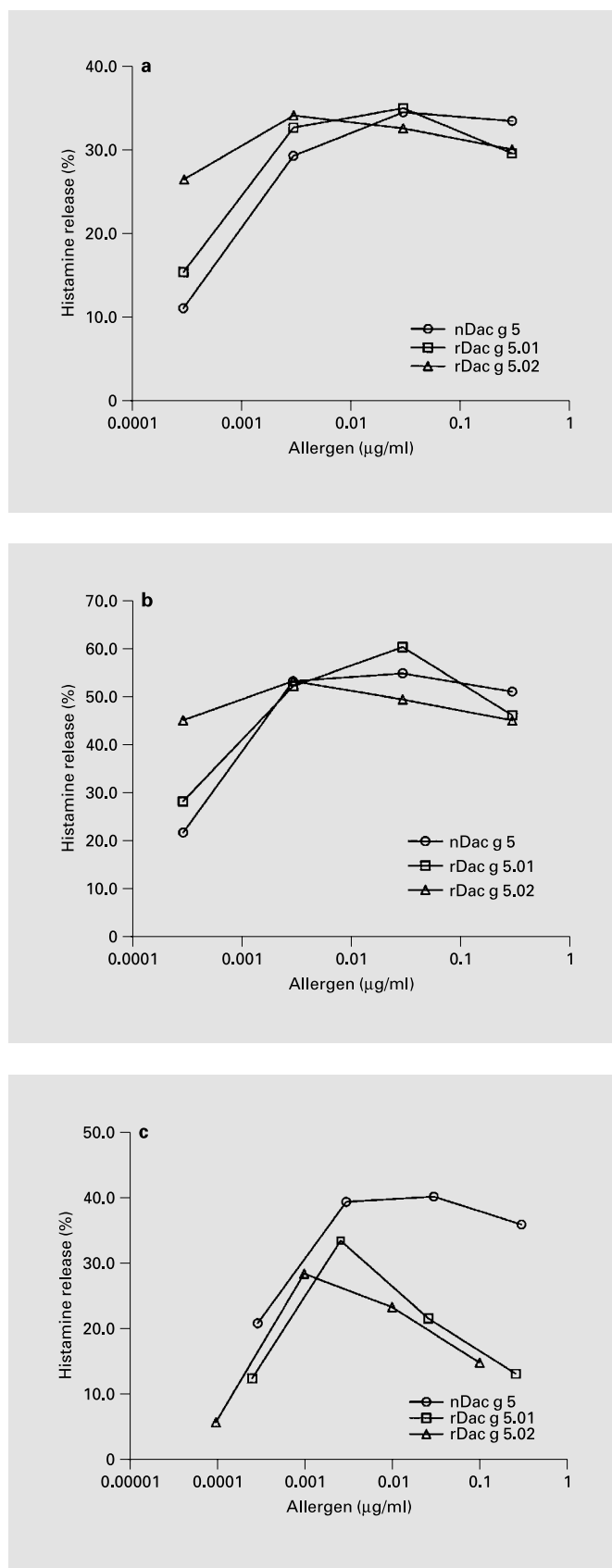
In a group 5 specific mAb-based sandwich ELISA, differences between nDac g 5, rDac g 5.01 and rDac g 5.02 were observed (fig. 6). Isoform 5.01 was recognized 3 times more efficiently than isoform 5.02, as calculated by parallel-line analysis. The difference observed between rDac g 5.01 and nDac g 5, in favor of the recombinant allergen, was not significant.

Discussion

Two different clones of Dac g 5 were expressed in *P. pastoris*. Both sequences differing at 4 positions were confirmed in independent PCRs and can therefore be designated as true isoforms, Dac g 5.01 and Dac g 5.02. They appeared on SDS-PAGE silver-stained gel as two protein bands as is also known from the natural protein, but with an apparent increase in molecular mass of approximately 10 kD. N-terminal sequencing showed a sequence homologous to that published for group 5 of *Dactylis* and *Lolium* [7, 16], indicating that the signal peptide was correctly cleaved from the secreted protein. Binding of Con A and sensitivity thereof to periodate treatment suggested that both recombinants were O-linked glycosylated, bearing in mind that no putative N-linked sites were present or described for group 5 allergens. O-linked glycosylation offers an explanation for the difference in apparent M_r between natural and recombinant allergen observed on SDS-PAGE. MALDI-TOF mass spectrometry confirmed a difference in mass, though the difference was smaller (2.5 kD instead of 10 kD). Most likely, this discrepancy is caused by slower migration on SDS-PAGE, as is often observed for glycoproteins. The two-dimensional structure obtained from CD spectrum measurements suggests minor differences between natural Dac g 5 and both recombinants; this is possibly due to the interference of the O-linked glycosylation with folding of the protein. The heterologous expression of naturally nonglycosylated proteins in *Pichia* can result in O-glycosylation and has been observed in our laboratory for several plant proteins. This possible modification has to be considered when choosing between prokaryotic and eukaryotic expression systems.

RAST analysis revealed that in limited group of patients (6/42) rDac g 5.02 is a less reactive isoform than rDac g 5.01 and nDac g 5. In spite of this rDac g 5.02 cannot be considered as a true hypoallergenic isoform. The difference between rDac g 5.01 and rDac g 5.02 is limited to 4 amino acid position which could be responsi-

Fig. 4. Histamine release assays. nDac g 5, rDac g 5.01, and rDac g 5.02. Both recombinants were compared to nDac g 5 for histamine-releasing capabilities with 6 grass pollen group 5 positive sera. Three patients are shown as examples. In patient 1 (a) and patient 2 (b) recombinants and nDac g 5 induce a similar response, but in patient 3 (c) a decreased response to rDac g 5.01 and especially to rDac g 5.02 was observed. The stripped cells (negative control) demonstrated an average release of 2% after allergen challenge (not shown).



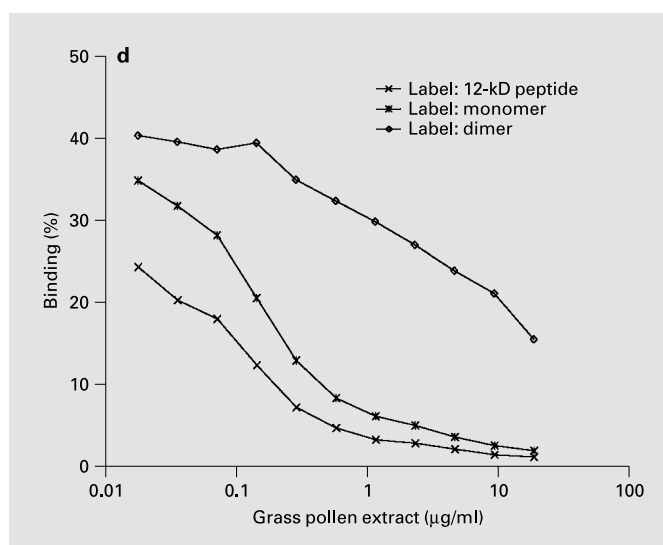
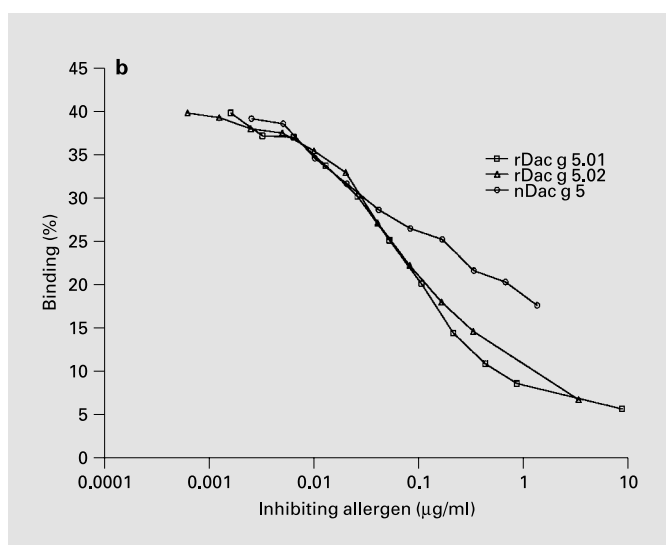
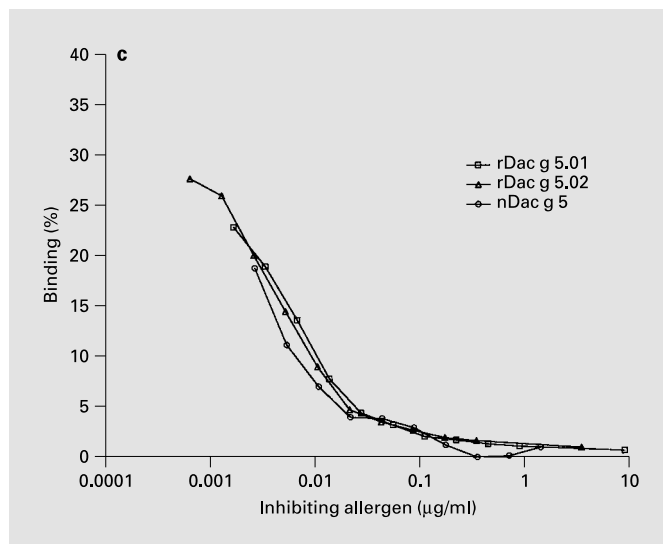
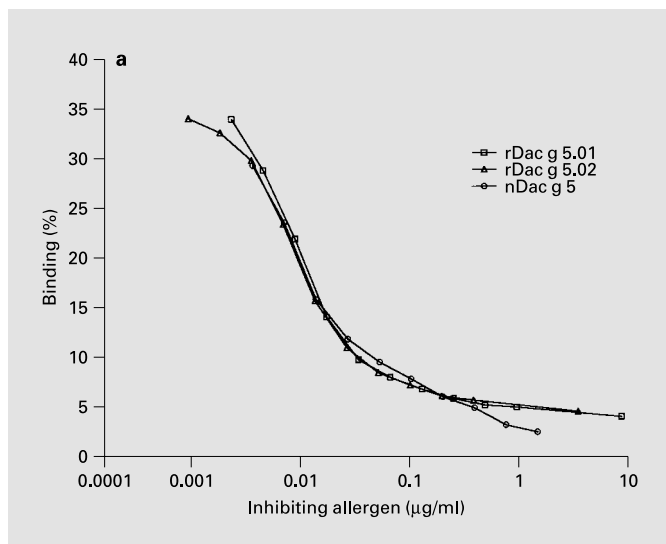
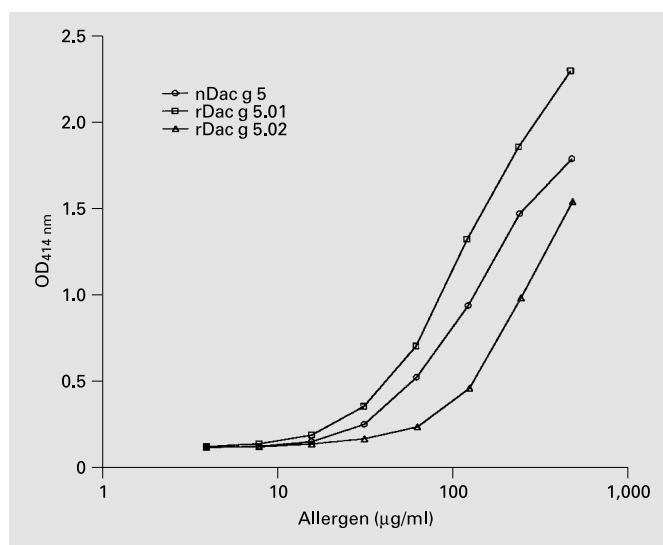


Fig. 5. **a** rDac g 5.01, rDac g 5.02 and nDac g 5 as the inhibiting allergens for nDac g 5 monomer label. **b** rDac g 5.01, rDac g 5.02 and nDac g 5 as the inhibiting allergens for nDac g 5 dimer label. **c** rDac g 5.01, rDac g 5.02 and nDac g 5 as the inhibiting allergens for nDac g 5 12-kD peptide label. **d** Total *Dactylis* extract was also used as inhibiting agent with all 3 labels, monomer, dimer and 12-kD peptide, respectively.

Fig. 6. Group 5 grass pollen mAb-based sandwich ELISA. rDac g 5.01 was recognized 3 times more efficient than rDac g 5.02. The difference observed between rDac g 5.01 and nDac g 5 did not reach significance.



ble for epitope alteration, although an influence of differences in glycosylation between both recombinants cannot be ruled out. A significant difference in immune reactivity of both isoforms was confirmed by mAb-based sandwich ELISA. Most human IgE antibodies as well as a polyclonal rabbit anti-serum did not distinguish between the two isoforms. Both rDac g 5.01 and rDac g 5.02 contain a cysteine, so far only described for two other group 5 allergens, Lol p 5c [40] and Phl p 5b [18], which were also found to be most homologous to our group 5 recombinant proteins. This cysteine could potentially be involved in the formation of the dimers which were observed on SDS-PAGE/immunoblot in our recombinant preparations. These experiments were performed under reducing conditions, showing that dimer formation is at least partially insensitive to the influence of reducing agents, thereby suggesting that some noncovalent interactions could be involved in dimer formation.

On the other hand, in the recombinants we did not observe the C-terminal breakdown or splice variant observed in purified natural Dac g 5 (12-kD band). Bufe et al. [19] described spontaneous breakdown of *E. coli* derived rPhl p 5b to an LMM C-terminal peptide. Possibly, O-glycosylation of our recombinants prevents this process of cleavage of the C-terminal part of group 5 allergens. Whether this cleavage is resulting in an active RNase, as suggested by Bufe et al. [18], still needs further confirmation.

Preliminary data on size exclusion chromatography with total *Dactylis* extract revealed possible dimerization of nDac g 5 and the presence of the 12-kD peptide, as judged by group-5-specific ELISA [manuscript in preparation].

In the amino acid sequence of both recombinants we found a second methionine at position 21. This potential start codon is in frame with the rest of the sequence, con-

tains a Kozak-like and GC-rich sequence upstream and could therefore be used as an alternative N-terminal. If translation started at this point, this would correspond to a molecular weight difference of about 2.3 kD. This is close to the difference between the main upper and minor lower band seen on SDS-PAGE analysis of rDac g 5. The same doublet is observed for natural Dac g 5, in this case with equal shares of both bands. Therefore, the use of a downstream start codon could also be the explanation for the naturally occurring doublet. The use of an alternative start codon has been described for other proteins [41]. In case of valyl-tRNA synthetase and threonyl-tRNA synthetase, the two versions were described to have a distinct function and intracellular destination [42]. An explanation for the limited share of the LMM band in the recombinants could be that translation from the second start codon results in absence of the α -factor signal sequence, thereby hampering secretion. The LMM band that is observed is probably explained by leakage from (dead) cells.

In conclusion, we have succeeded in expressing a stable recombinant version of an important major group 5 grass pollen allergen, Dac g 5, in the yeast *P. pastoris*. Analysis of the immune reactivity of two isoforms confirmed earlier observations for Bet v 1 [43] and Der p 2 [44] that minor sequence differences can have significant impact on antibody recognition.

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