

The Major Grass Pollen Group 5 Allergen from *Dactylis glomerata* and Its C-Terminal Split Product Both Behave as Dimers: Implications for Allergen Standardization

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Key Words

Dactylis glomerata · Group 5 allergens · Dimers · Doublet · C-terminal split product · Allergen standardization protocols

Abstract

Background: On SDS-PAGE grass pollen group-5 allergens migrate as a doublet with an apparent molecular mass (M_r) of 25 kDa. Immunoblot analysis revealed additional group 5 reactivity at double and half this M_r . The aim of this study was to investigate these group 5 molecular entities and to compare their allergenicity and behavior in quantitative immunoassays. **Methods:** Group-5-specific monoclonal antibodies were produced and used for the development of a group-5-specific sandwich ELISA. Affinity-purified Dac g 5 was separated by SDS-PAGE/Western blotting; individual bands were analyzed by N-terminal sequencing. Size exclusion chromatography (SEC) in conjunction with group-5-specific ELISA, competitive RIA and RAST inhibition were used to analyze the size distribution of Dac g 5. Basophil histamine release assays were used to assess biological activity. **Results:** The lower band of the typical group 5 doublet

was identified as a truncated form lacking the typical group 5 N-terminus AD^L/A^GY, observed in the upper band. The 12-kDa peptide was shown to be the C-terminal half of Dac g 5 (amino acid 127 onwards). SEC in conjunction with competitive RIA revealed that around 45% of Dac g 5 is represented by the 12-kDa peptide. Both the C-terminal half and the whole allergen dimerize under nondenaturing conditions. In competitive RIA and RAST inhibition both forms are equally well detected. In contrast, the half molecule is poorly recognized in sandwich ELISA and displays negligible biological activity in basophil histamine release tests with purified IgE. **Conclusions:** These observations stress the need to evaluate the performance of allergen standardization protocols in detail, with special attention to allergen size distribution.

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Introduction

Sensitization to grass pollen allergens is one of the most common causes of inhalant allergies. To date, grass pollen allergens are divided into 11 groups [1–5], of which group 1 and group 5 have been identified as the most important.

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Several group 5 molecules have already been purified and/or cloned [6–14] and are described as a group of non-glycosylated, highly homologous proteins with an apparent molecular mass (M_r) on SDS-PAGE of 25–33 kDa [15–17]. By immunoblot analysis, group 5 allergens were shown to migrate as a doublet around this M_r , and both bands showed IgE-binding capacities [4, 5, 18]. Matthiesen and Lowenstein [4] were the first to designate this doublet of natural Phl p 5 as the a-form and b-form with an approximate M_r of 32 and 29 kDa, respectively. N-terminal microsequencing of these two bands revealed some amino acid exchanges, but both started with the characteristic group 5 N-terminal sequence (AD^L/A^GY). These results were confirmed by Petersen et al. [19] although slightly higher M_r were reported for both forms. Other studies, however, suggested the existence of N-terminally truncated group 5 allergens [16, 20]. Another characteristic of group 5 allergens was revealed by immunoblot analysis where group 5 activity was also found at approximately double its M_r (~49 kDa) and half its M_r (~12 kDa) [5, 14, 21, 22]. The nature of these group-5-related structures has not yet been completely elucidated. Matthiesen and Lowenstein [4] concluded from size exclusion chromatography (SEC) experiments that Phl p 5 exists in two molecular forms, one of 47 kDa and one of 25 kDa. On SDS-PAGE, the 47 kDa fraction appeared as a doublet around 25 kDa, suggesting to be a mixture of Phl p 5a and Phl p 5b. The 25-kDa fraction had an apparent M_r of 12 kDa and was thought (but not confirmed) to be a degradation product [5]. Another indication for the existence of a group 5 degradation product was reported by Bufe et al. [23]. They observed the spontaneous appearance of a C-terminal peptide of around 12 kDa after Phl p 5b expression in *Escherichia coli*. From X-ray crystallographic analysis it was concluded that this C-terminal peptide crystallized as a dimer. Upon resolubilization, this dimer showed similar allergenicity compared with the whole allergen [23, 24]. Recently, this C-terminal dimer was shown to consist of four α -helices. The authors also pointed to significant internal homology between the C- and the N-terminal halves of Phl p 5, and suggested similar structural features for both halves, possibly facilitating dimerization. Bufe et al. [23] have also demonstrated that IgE antibodies affinity-purified from the C-terminal peptide also recognize the N-terminal half of the molecule [24].

Grass pollen extracts are widely used for allergy diagnosis (e.g. skin prick test, radioallergosorbent test; RAST) and allergen-specific immunotherapy. Variability in the composition of such extracts is a problem inherent in bio-

logical products. Purified major allergens were already proposed and tested as alternatives for those extracts several decades ago [25, 26]. Although molecular biology has facilitated the production of recombinant versions of major allergens, recombinant products are not yet available on the market to replace current extract-based products. It is thus important that major allergens present in grass pollen are consistently measured in standardization protocols. Over the past decades immunoassays based on monoclonal antibodies (mAb) have been developed for most major allergens. Those assays are powerful tools for standardization purposes. In some cases though, it has been demonstrated that the assays are too specific, failing to detect individual isoforms [27–30].

It was the aim of this study to characterize the nature of the heterogeneous mixture of Dac g 5 molecular entities (doublet, dimers and 12-kDa peptide). To the best of our knowledge it is not known whether a so-called 'a'- and 'b'-form comparable to Phl p 5 exist in *Dactylis* group 5 allergens nor are the characteristics and allergenicity of those different molecular entities known. Furthermore, different standardization assays were compared to investigate their suitability for standardization of Dac g 5 molecules in crude and purified preparations.

Materials and Methods

Purification of Group 5 Allergens

Group 5 allergens were affinity-purified, either directly from pollen extracts or from Phenyl Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden) retarded fractions, with a group-5-specific mAb (4B1) as described elsewhere [18]. In the latter case, pollen extracts were incubated with Phenyl-Sepharose in 1 mol/l ammonium sulfate. Under these conditions group 5 allergens have been reported to bind to Phenyl Sepharose [18]. Elution of group-5-enriched fractions was achieved with water, and was followed by dialysis against PBS. Recombinant Dac g 5.01 was affinity-purified from *Pichia pastoris* culture supernatant as described elsewhere [14].

Monoclonal Antibodies

Balb/c and B-10 mice (Charles River, Germany) were immunized subcutaneously with 10 μ g affinity-purified Phl p 5 in Montanide adjuvant ISA-50 (Seppic, Paris, France). The immunization was followed by two subcutaneous (4 and 8 weeks after immunization, respectively) and one intravenous booster (administered 4 days before the removal of the spleen) with 10 μ g Phl p 5 in Montanide adjuvant. Hybridization was performed according to the method of Köhler and Milstein [31, 32] with some modifications [33]. Antibody-producing hybridomas were screened by a radioimmunoassay with Sepharose-coupled goat-anti-mouse IgG (GoMo17, Sanquin, Amsterdam, The Netherlands) and ¹²⁵I-labeled affinity-purified Lol p 5. By using Lol p 5 instead of Phl p 5, screening was deliberately

biased towards cross-reactive group 5 mAbs. Cloning was performed by repeated limiting dilution. This procedure resulted in 6 Balb/c-derived mAbs (2C8, 11D11, 13D9, 18B11, 14C2 and 19F9) and 2 B-10-derived mAbs (1G6 and 8E2).

Group-5-Specific mAb-Based Sandwich ELISA

A specific mAb-based sandwich ELISA was developed for quantification of group 5 allergens in grass pollen extracts. The ELISA was developed with three mAbs that were selected on the basis of their recognition profile of group 5 isoforms on immunoblot after separation of a grass pollen extract by 2D electrophoresis [34]. These 2D profiles showed that mAb 14C2 was able to bind to acidic as well as basic isoforms, in contrast to 13D9 and 8E2 which were shown to bind with a preference for the basic or acidic isoforms, respectively. mAb 14C2 was chosen as capturing antibody with a coating concentration of 2 µg/ml in coating buffer (50 mM sodium carbonate, pH 9.6). Both 13D9 and 8E2 were biotinylated and used as detecting antibodies each with a concentration of 125 ng/ml in blocking buffer (PBS, 1% BSA, 0.05% Tween-20). Dilutions of grass pollen extracts and affinity-purified samples were also made in blocking buffer. Streptavidin-horseradish peroxidase (Amersham Biosciences UK limited, Little Chalfont, UK) was used as a conjugate, diluted 1:2,000 in blocking buffer. 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 peroxidase conjugate, which was incubated for 30 min. Color development with ABTS [2,2'-azinobis(3-ethylbenz-thiazolinesulfonic acid), Sigma, St. Louis, Mo., USA] 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) and the absorbance measured at 414 nm.

In addition, a homologous sandwich ELISA was developed using mAb 13D9 as catching (coat 2 µg/ml) and biotinylated detecting antibody (250 ng/ml). The assay was further performed as described above.

SDS-PAGE/Silver Staining and Immunoblotting

Proteins were separated by SDS-PAGE (4–12%) (Novex, San Diego, Calif., USA) as described by the manufacturer (under reducing or nonreducing conditions) and silver stained according to Bexel biotechnology silver staining kit protocol (Bexel Biotechnology, Union City, Calif., USA). Western blotting was performed by transferring the proteins to 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 mouse mAbs 4B1 [18], 14C2, 13D9 and 8E2 or polyclonal rabbit antibodies [18] and 3 human sera with group-5-reactive IgE antibodies. After washing, the blots were incubated overnight with ¹²⁵I-labeled goat anti-mouse IgG (GoMo17), sheep anti-rabbit IgG (HoRb17) or sheep anti-human IgE antibodies (SH25, Sanquin), respectively. After washing, blots were exposed to an autoradiographic film (Eastman Kodak Company, Rochester, N.Y., USA).

N-terminal Sequencing of Dac g 5

Affinity-purified nDac g 5 was separated by SDS-PAGE and electro-blotted on polyvinylidene difluoride membrane. Blots were stained with 0.1% Coomassie R-250 in 50% methanol. The bands corresponding to the Dac g 5 doublet (around 25 kDa) and the ~12-kDa peptide were excised and sequenced on a Perkin Elmer/Applied Biosystems 476A gas-phase sequencer.

Size Exclusion Chromatography

SEC was performed on an ACA 54 (Biosepra SA, Cergy-Saint-Christophe, France) SEC column equilibrated in PBS (column volume ~450 ml, diameter 24 mm, height 1,030 mm). *D. glomerata* extract, or a fraction thereof enriched in group 5 allergens by prepurification on a Phenyl Sepharose column, was loaded on to the SEC column in a volume of 5 ml in PBS (~2 mg/ml). Elution of the column was subsequently performed in PBS and fractions of ~2 ml (40 droplets) were collected.

In order to determine the molecular size(s) of Dac g 5, the ACA 54 column was calibrated with the following proteins in PBS: ribonuclease A (13.7 kDa), chymotrypsin (25 kDa), ovalbumin (43 kDa), albumin (67 kDa) and dextran blue (2,000 kDa) (LMW gel filtration calibration kit, Amersham Pharmacia Biotech Inc., Piscataway, N.J., USA). Based on protein determinations (Pierce) of all fractions, the following elution pattern was observed: dextran blue (~155 ml), albumin (~182 ml), ovalbumin (~210 ml), chymotrypsin (~330 ml) and ribonuclease A (~378 ml). The M_r were calculated on the basis of the elution volumes of Dac g 5 using a formula obtained through extrapolation of the calibration proteins (trendline calculated by logarithmic regression: $y = -63.829 \ln(x) + 392.78$, $R^2 = 0.9271$).

Radio-Labeling

Purified nDac g 5 (25 µg) was radio-labeled with ¹²⁵I using the chloramine-T method. Radio-labeled allergen and free iodine were separated by size exclusion chromatography (ACA54). The ¹²⁵I-labeled nDac g 5 gave an elution pattern of three distinct peaks with decreasing M_r . The third peak (C-terminal peptide) was used in competitive RIA [14].

Competitive RIA

In a competitive RIA [35, 36], 50 µl of a rabbit anti-group 5 antibody (1:3,000) [18] was preincubated for 2 h at room temperature with 50 µl of serial dilutions of the inhibitor (nDac g 5 and fractions obtained by SEC). After incubation, 250 µl Protein A Sepharose (2 mg/ml; Amersham Pharmacia Biotech AB) and 50 µl of ¹²⁵I-labeled nDac g 5 were added, followed by incubation overnight at room temperature. After washing, bound radioactivity was measured using a gamma counter. Results were expressed as percentage inhibition relative to an uninhibited value. For the uninhibited value, polyclonal rabbit antibodies were preincubated with PBS-AT instead of inhibiting allergen.

RAST Inhibition

RAST inhibition was performed as described previously [37]. Preincubation (2 h) of 50 µl of a grass-pollen-positive serum pool ($n > 100$, > 3 IU/ml) with 50 µl of serial dilutions of nDac g 5 (1 ng/ml–10 µg/ml) or SEC fractions (0.37 ng/ml–30 µg/ml total protein), was performed before addition of nDac g 5 coupled to Sepharose (100 µg–100 mg) [14]. After incubation overnight, unbound material was washed away and 50 µl of ¹²⁵I-labeled sheep anti-human IgE (SH 25, Sanquin) were added. After incubation overnight and washing, percent binding of radioactivity was measured using a gamma counter. The results were expressed as international units per milliliter. They were calculated using a standard curve of serial dilutions of a human/mouse chimeric IgE antibody directed to Der p 2 and Sepharose-coupled rDer p 2 [38].

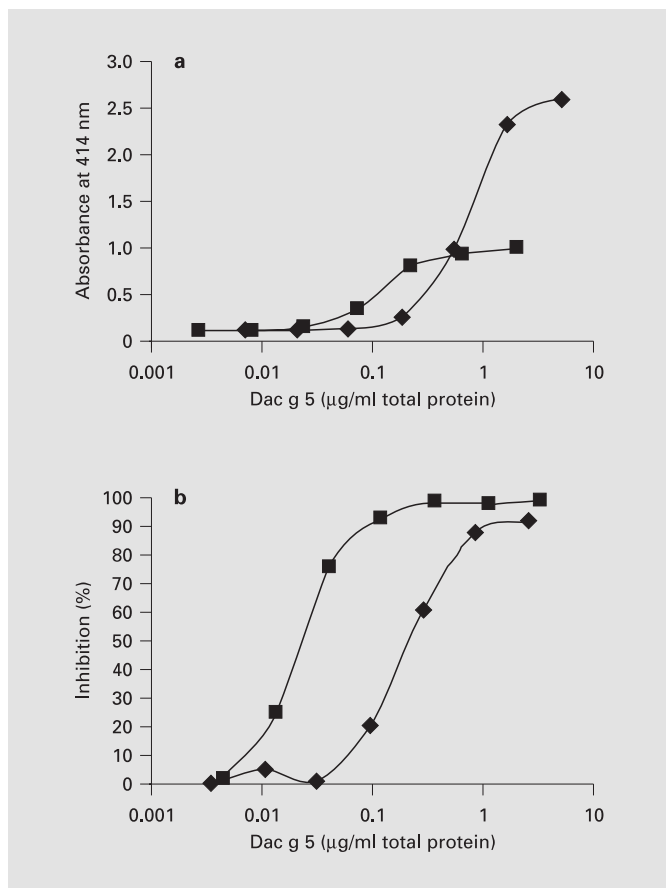


Fig. 1. a, b Group-5-specific mAb-based sandwich ELISA and group-5-specific competitive RIA. **a** Group-5-specific ELISA with Phl p 5 mAbs cross-reactive with *D. glomerata* extracts (◆) and natural affinity-purified Dac g 5 (■). **b** Competitive RIA with a group-5-specific rabbit polyclonal antibody. Dac g total grass pollen extract (◆) and affinity-purified nDac g 5 (■) were used as inhibitors of ¹²⁵I-labeled Dac g 5 (C-terminal peptide) label.

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 [39, 40]. Subsequently, cells were re-sensitized with affinity-purified group-5-specific IgE. To this end, serum was incubated (2 h rotation) with rDac g 5.02 coupled to Sepharose. The Sepharose was subsequently washed with PBS to remove unbound material and the group-5-specific IgE was stripped from the Sepharose with 0.1 M glycine, pH 2.5. The IgE-containing fractions were immediately neutralized by addition of 1 M Tris-HCl pH 8.5. A RAST was performed to measure the yield of group-5-specific IgE in affinity-purified fractions. Histamine release was tested with purified nDac g 5 and SEC fractions representing the high- and low-M_r peaks of nDac g 5. Liberated histamine was measured essentially by the fluorometric method described by Siraganian [41]. The protocol was approved by the medical ethical committee of the Amsterdam Medical Center under project No. MEC97/030.

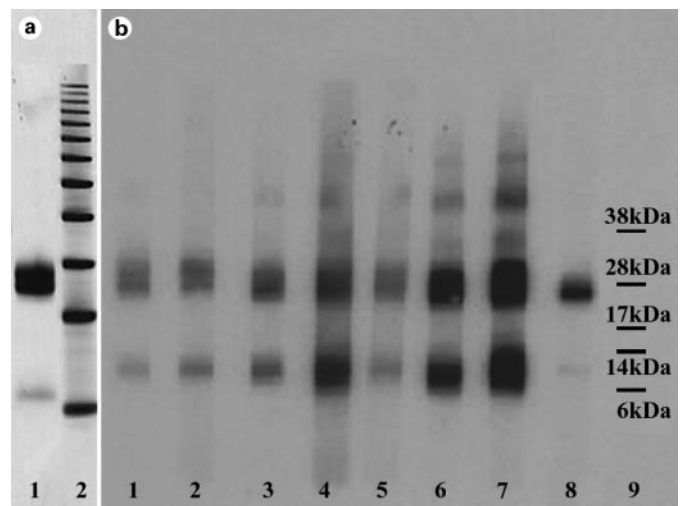


Fig. 2. a, b SDS-PAGE/silver staining and immunoblot with affinity-purified nDac g 5. **a** Lanes 1 and 2 represent SDS-PAGE/silver staining of affinity-purified nDac g 5 and a 10-kDa protein ladder, respectively. **b** Immunoblot performed with 3 group-5-positive sera 155, 166 and 200, in lanes 1–3, respectively. Lanes 4, 6–8 were blotted with group-5-specific mAbs 4B1, 14C2, 13D9 and 8E2, respectively. Lane 5 was blotted with a group-5-specific rabbit polyclonal antibody and lane 9 represents the Seebule plus 2 protein marker (Novex).

Protein Concentrations

Protein concentrations were determined, using the BCA method as described by the manufacturer (Pierce, Rockford, Ill., USA).

Statistical Analysis

Parallel line analysis was performed on ELISA and competitive RIA results to calculate the specific group 5 allergen content in unknown samples.

Results

Group-5-Specific mAb-Based Sandwich ELISA

An mAb-based sandwich ELISA was developed that detected group 5 allergens in *D. glomerata* extracts, illustrating that the selected anti-Phl p 5 monoclonals were cross-reactive (fig. 1a). Surprisingly, when affinity-purified Dac g 5 (either isolated from whole extract or Phenyl-Sepharose-enriched fractions) was tested, a much lower plateau was reached at higher allergen concentrations than with pollen extract, and the linear part of the curve was shorter. This discrepancy was not observed in the group-5-specific competitive RIA (fig. 1b).

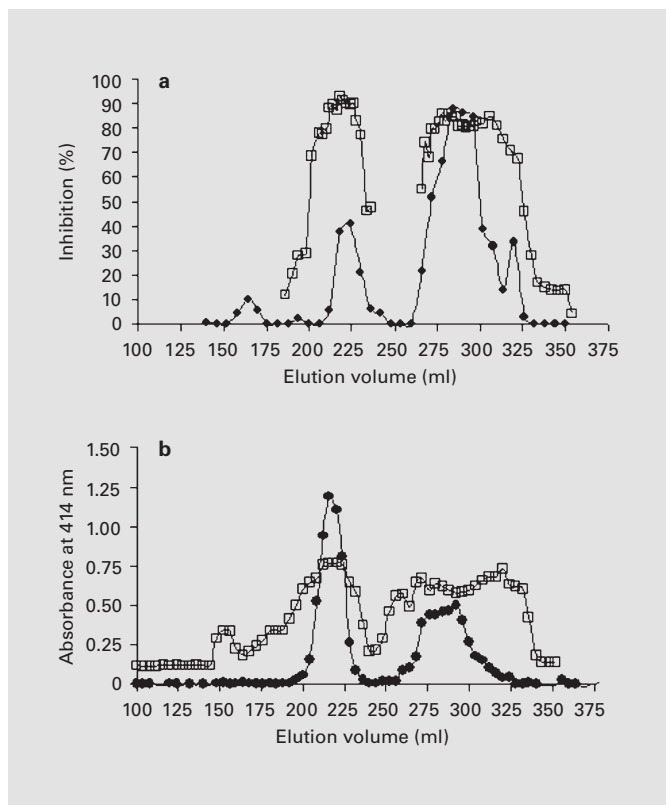


Fig. 4. a, b Competitive RIA and sandwich ELISA. **a** Competitive RIA on ACA 54 fractions of total extract (□) and Phenyl-Sepharose-enriched extract (●). **b** Sandwich ELISA on ACA 54 fractions of total extract (□) and Phenyl Sepharose enriched extract (●).

The N-terminal sequence of the 12-kDa fragment gave a 100% match with the reported protein sequences of rDac g 5.01 and rDac g 5.02 [14], starting at amino acid 127 of the mature recombinant protein: AEEVPAAKI-PAGEL (fig. 3).

SEC: Dac g 5 Forms Dimers

D. glomerata extract as well as a fraction thereof prepurified by hydrophobic interaction chromatography (Phenyl Sepharose), were separated by SEC and fractions were analyzed by competitive RIA and sandwich ELISA. In both immunoassays the existence of a 49-kDa and a 30-kDa form was observed (fig. 4a, b). The analysis revealed that the ratio between these forms shifted in favor of the lower- M_r variant by purification over Phenyl Sepharose. Furthermore, the ELISA showed a preference for the 49-kDa form compared with the competitive RIA. To further characterize the ratio of the 49- and 30-kDa fractions, affinity-purified Dac g 5 (from whole extract) was frac-

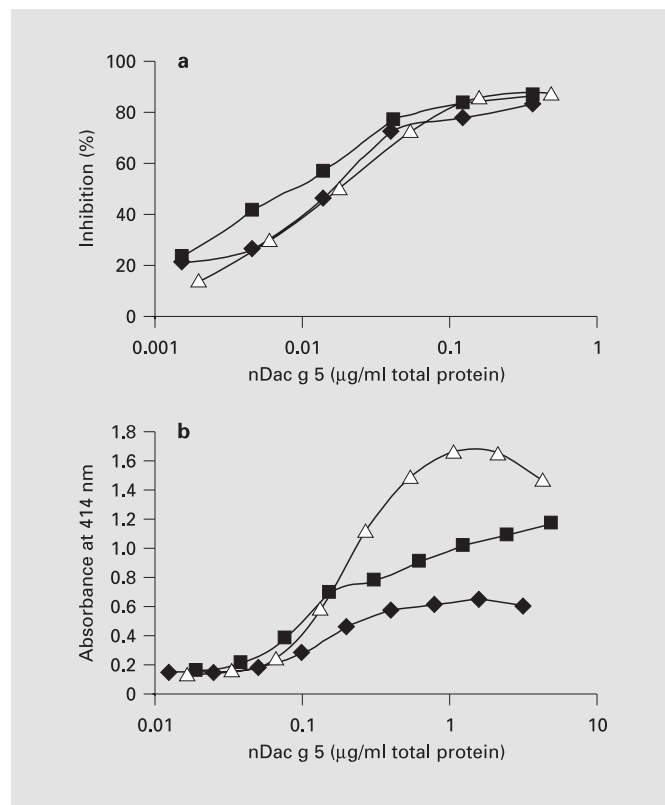


Fig. 5. a, b Group-5-specific competitive RIA and group-5-specific mAb-based sandwich ELISA with ACA 54 fractions. **a** Competitive RIA comparing the inhibiting potencies of affinity-purified nDac g 5 (■) and SEC fraction 56 of the 49-kDa peak (△) and fraction 110 of the 30-kDa peak (◆) with ^{125} I nDg 5 (C-terminal peptide) label. **b** Sandwich ELISA comparing the dose-response curves of affinity-purified nDac g 5 (■), SEC fraction 56 of the 49-kDa peak (△) and SEC fraction 110 of the 30-kDa peak (◆).

tionated by SEC. Dose-response curves of fractions from the 49- and the 30-kDa peak overlapped in competitive RIA (fig. 5a). In contrast, the ELISA did not efficiently detect the 30-kDa peak (fig. 5b). Therefore, the ratio between the 49- and 30-kDa molecular forms of Dac g 5 were based on the results obtained by competitive RIA. Dac g 5 in total extract was calculated to be composed of 55% of the 49-kDa and 45% of the 30-kDa form. Almost the same ratio was observed for affinity-purified Dac g 5 (56% vs. 44%). However, after purification over Phenyl Sepharose, the share of the 30-kDa variant appeared to increase to 92%.

Several SEC fractions including both M_r variants were analyzed by SDS-PAGE/silver staining under reducing (fig. 6a) and nonreducing conditions (fig. 6b). Reduction did not have significant impact on the apparent M_r of sep-

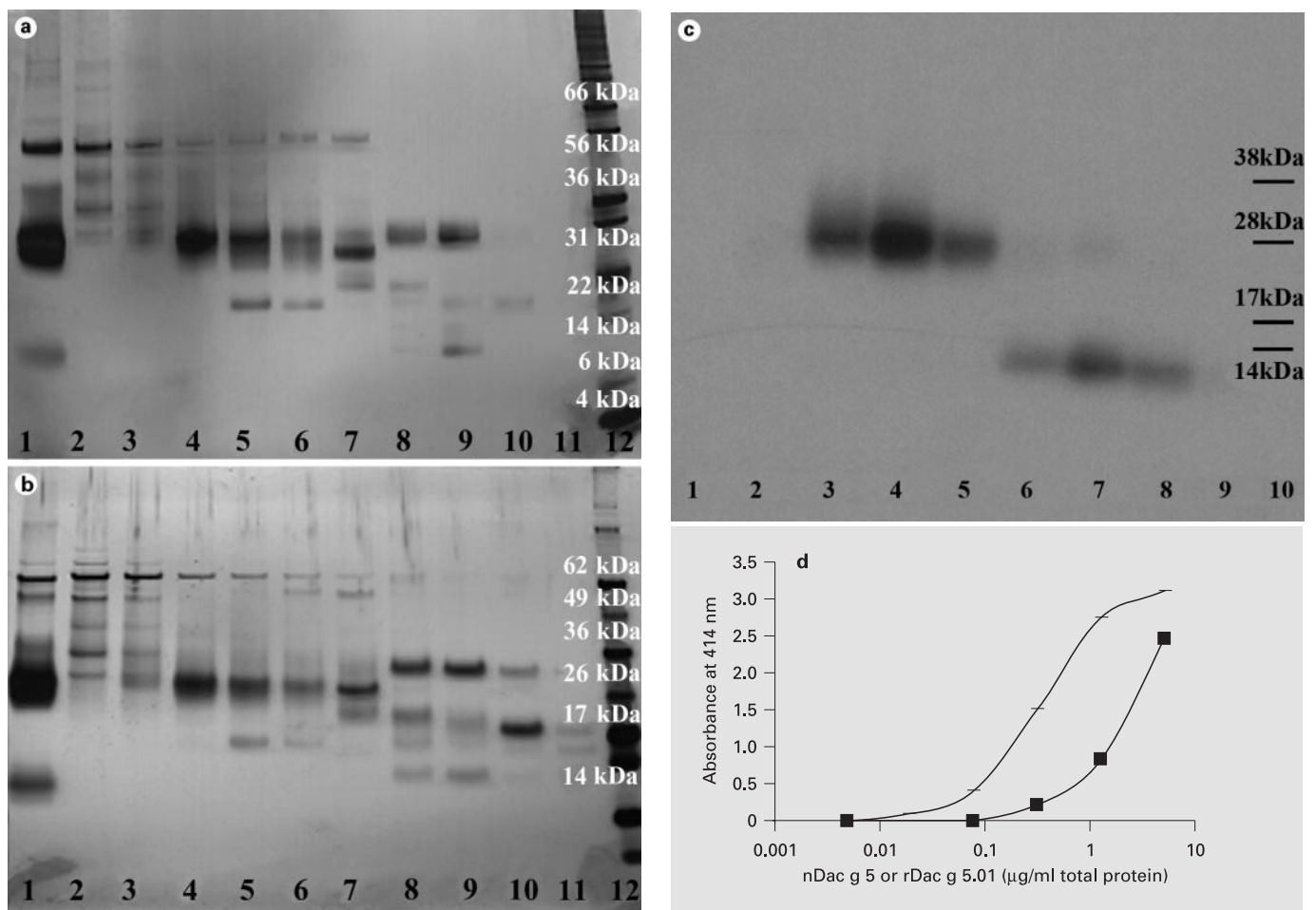


Fig. 6. a–d SDS-PAGE/silver staining, immunoblot of SEC fractions and homologous sandwich ELISA. **a** SDS-PAGE silver staining (reducing conditions) of fractions collected after SEC. Lanes 1–10 represent affinity-purified Dac g 5, SEC fractions collected at 162, 182, 212, 222, 242, 282, 302, 312 and 332 ml and a mark 12 protein ladder (Novex), respectively. **b** SDS-PAGE silverstaining (nonreducing conditions), representation of nDac g 5 and SEC fractions as

described in **a**. Lane 12 represents the See blue plus 2 protein marker (Novex). **c** Immunoblot (mAb 4B1) of SEC fractions collected at 162, 182, 212, 222, 242, 282, 302, 312 and 332 ml shown in lanes 1–9, respectively. Lane 10 represents the See blue plus 2 protein marker (Novex). **d** Homologous group-5-specific sandwich ELISA with nDac g 5 (■) and rDac g 5.01 (—).

arated fractions, suggesting that disulfide bridges are not instrumental in dimerization. Immunoblot analysis revealed that the fractions containing the 49-kDa form correspond to a protein band exclusively detected at an apparent M_r of approximately 25 kDa. The 30-kDa form corresponds to a protein band exclusively detected at 12 kDa under reducing conditions (fig. 6c). This suggests that both forms of Dac g 5 observed in SEC are dimers of the complete molecule and of the C-terminal half molecule, respectively. Further support for dimerization was obtained by detection of Dac g 5 in a homologous mAb-based sandwich ELISA (fig. 6d).

Allergenic Potency of Different Molecular Forms of Dac g 5

In RAST inhibition with a group-5-reactive serum pool, 49- and 30 kDa fractions showed very similar potencies to inhibit the binding of IgE to affinity-purified nDac g 5 (fig. 7a). In contrast, the biological activity of the 30 kDa peak was shown to be minor in comparison with the 49-kDa peak when affinity-purified IgE ($n = 2$) was used to stimulate the basophils (fig. 7b).

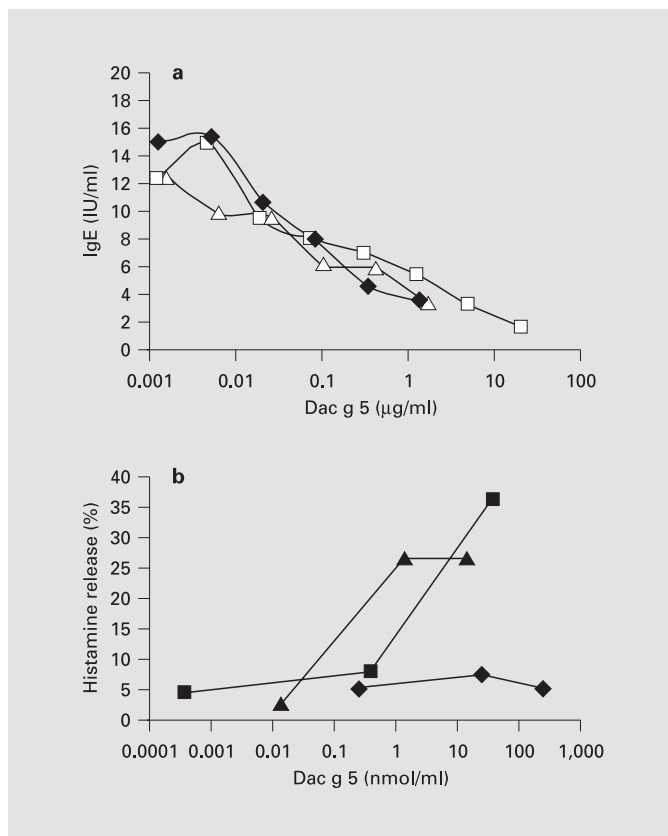


Fig. 7. a, b RAST inhibition and histamine release assay. **a** RAST inhibition of affinity-purified nDac g 5 (□), fraction 56 from the 49-kDa peak (△) and fraction 110 from the 30-kDa peak (◆) are shown. The amount of specific Dac g 5 activity in the tested samples (comp. RIA) is displayed as µg/ml. Results are expressed as IU/ml IgE. **b** Histamine release assay: Affinity-purified nDac g 5 (■), fractions 56/58 of the 49-kDa peak (▲) and fractions 95/110 of the 30-kDa peak (◆). The amount of specific Dac g 5 is expressed in nmol/ml. The release was calculated as percentage from total histamine release. The stripped cells (negative control) showed a release of 3.3% (not shown).

Discussion

The present study has shown that *D. glomerata* pollen extract contains almost identical quantities of the whole Dac g 5 allergen and of its C-terminal half. Physicochemical purification strategies for group 5 allergens involving hydrophobic interaction chromatography [18] seem to enrich in the C-terminal half molecule. This suggests that a fraction of the whole molecule of Dac g 5 binds with higher affinity to Phenyl Sepharose than the C-terminal fraction and may not efficiently elute from the column with water. Whether a fraction of Dac g 5 is still bound to

the Phenyl Sepharose that might be eluted with more hydrophobic buffers (e.g. ethylene glycol) was not investigated. Indeed, Suck et al. [42] have reported that the a-form of Phl p 5 is retained on Phenyl-Sepharose when elution is performed with water. It is therefore possible that a similar phenomenon occurs for *D. glomerata* and that this results in enrichment for the C-terminal peptide.

Our study has clearly shown that both the whole molecule and its C-terminal peptide behave as dimeric structures under nondenaturing conditions as was shown by SEC and homologous ELISA. The mechanism for dimerization is still unknown, but the absence of a difference on SDS-PAGE between reducing and nonreducing conditions suggests that disulfide bridges are not involved. Although, a cysteine has been reported in the cloned Dac g 5 sequence. Bufer et al. [43] suggested that a tyrosine present in the rPhl p 5b C-terminal peptide might be involved in dimerization (position 52 of the C-terminal fragment).

The existence of breakdown products of roughly half the molecular mass has been reported before [5, 18, 44], but this is the first time that the exact nature of this group 5 C-terminal peptide (start at aa 127) has been revealed for the natural allergen of Dac g 5. For recombinant Phl p 5, Bufer et al. [22, 23] reported that the C-terminal half of the allergen appears spontaneously after expression in *Escherichia coli*, and that this peptide has RNase activity. Whether the C-terminus of Dac g 5 is generated posttranslationally, by proteolytic activity, or whether the peptide has its own gene is not known. So far, there are no reports showing that the natural allergen is degraded to its C-terminal half, except for two recombinant Phl p 5 products [23, 45]. Maglio et al. [45] reported a time-dependent degradation of the N-terminus of rPhl p 5a. All group 5 allergens cloned so far, a-, b- and c-forms, have very similar N-termini starting with the typical AD^L/AGY sequence. The difference in length of their primary sequences is explained by gaps in four hypervariable regions of the molecule further downstream (framed in black in figure 3). Whether the existence of clones with variable length is the explanation for the observed doublet of group 5 allergens is not really clear. Some N-terminal sequence analyses of natural group 5 allergens have shown that both bands start with an identical sequence, supporting that the difference in apparent molecular mass has to be found elsewhere in the molecule [5, 19, 46]. However, our study with natural Dac g 5 suggests that the lower band of the typical group 5 doublet is a N-terminally truncated form of the allergen, as identified by N-terminal sequencing. Earlier reports confirm the existence of group

5 allergens starting slightly downstream of the usual AD^L/AGY N-terminus [16, 20].

It was shown by RAST inhibition that the C-terminus of Dac g 5 covers virtually the whole epitope spectrum of the anti-Dac g 5 response. The observation by Bufer et al. [24] that IgE antibodies affinity-purified from the C-terminal part of rPhl p 5b are also able to recognize the N-terminal part of the molecule is concordant with this observation. In our study, the C-terminus of nDac g 5 showed lower biological activity in histamine release assays than the whole molecule. It cannot be completely ruled out that the use of affinity-purified IgE is involved in the decreased histamine release. The lower biological activity of the C-terminal peptide of Dac g 5 is in contrast with the histamine-releasing potency of the C-terminus of rPhl p 5b, which was similar to the whole recombinant molecule [23]. If the decreased biological activity of the C-terminus of nDac g 5 is confirmed with additional sera, this implies that the C-terminal half of Dac g 5 might be used as a hypoallergenic structure in group-5-specific immunotherapy.

Standardization of allergenic extracts is an important part of quality control procedures for allergen manufacturers. This study has demonstrated that RAST inhibition detects biologically active and less active forms of Dac g 5 equally well. The same was true for a competitive RIA

using radiolabeled C-terminus of Dac g 5. In contrast, a mAb-based sandwich ELISA demonstrated a strong preference for the biologically active dimer of the complete allergen. These differences are important observations for allergen standardization.

In summary, this study has clearly demonstrated that Dac g 5 has various molecular appearances. The doublet characteristic for group 5 allergens exists as a dimer and is constituted of two proteins with different M_r and N-terminus. The 12-kDa peptide present in *D. glomerata* extracts also exists as a dimer and was found to represent the C-terminus of Dac g 5. Both the whole molecule and the C-terminal peptide were shown to have diverging biological activities and were variably detected in immunoassays for standardization. Heterogeneity is not unique to Dac g 5. Therefore, we suggest to include the size exclusion profiles of allergens in the process of evaluation and validation of assays for allergen standardization.

Acknowledgements

We thank Fridolin van der Lecq and others for their quick and excellent work on the protein sequences (Sequentie centrum, Utrecht, The Netherlands). This study was financially supported by Stallergènes SA, Altadis, ANVAR and CNRS.

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