

Application of reversed-phase high-performance liquid chromatography in the purification of major allergens from grass pollen

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Received 13 September 1995; revised 20 November 1995; accepted 12 January 1996

Abstract

Group 1 and 5 allergens of different grasses possess similar physicochemical parameters (molecular weight, *pI*) and therefore separation with conventional chromatographic methods (gel filtration, ion exchange chromatography) is difficult or impossible to achieve. In this paper we describe the isolation of biologically active group 1 and 5 allergens from extracts of *Lolium perenne* and *Phleum pratense* by means of reverse phase chromatography on HPLC. The chromatograms showed very different retention times for group 1 (*Rt* 19.1–20.5 min) and group 5 (*Rt* 24.3–26.3 min) containing fractions. In addition, this technique is suitable for the separation of group 5 allergens into 5a and 5b subgroups and for the estimation of the amounts of allergen (groups 1 and 5) in the different extracts.

Keywords: Grass extract; Allergen purification; Chromatography, reverse phase high performance liquid; Chromatography, group 1 and group 5 allergens

1. Introduction

In recent years many groups have reported the isolation and characterization of grass pollen allergens (reviewed by Singh et al., 1990). The pioneering work of Johnson and Marsh (1965) led to the isolation of various allergenic components from rye-grass pollen which were shown to be immunochemically distinct. Since then the physicochemical param-

eters (molecular weight, *pI*) of several allergens have been determined. Because of the high cross-reactivity between pollens of different species of grass, similar allergens have been classified into the same allergen groups (Marsh et al., 1988; King et al., 1994). The most important allergens in grass pollen extracts are those of groups 1 and 5 with a prevalence of reactive IgE antibodies of > 90%. Both groups of allergens can be characterized by immunochemical procedures and, very recently, by molecular cloning techniques (Breitenbach et al., 1990). Group 1 and 5 allergens of different grasses possess similar molecular weights (range 25–35 kDa) and

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pIs (range 4.8–7.0), and therefore separation with conventional chromatographic methods (gel filtration, ion exchange chromatography) is difficult or impossible to achieve. However, the preparation of highly purified allergens of a defined group is often necessary, e.g. for B and T cell epitope studies. Affinity chromatography using suitable 'grass group specific' mAbs may be an alternative technique for the isolation of certain allergens (Jung et al., 1989; Mourad et al., 1986), but only a few laboratories possess suitable mAbs. Moreover the elution conditions (acid pH or high salt concentration) can result in loss of allergenic activity. The application of gene technology represents a new way to produce allergens (Laffer et al., 1994), but a prerequisite for the use of such allergens is that the natural and recombinant proteins possess comparable functional properties, e.g. ability to stimulate allergen-specific T cells. At present, several recombinant allergens have been synthesized, but their IgE-binding capacity is often less than that of corresponding natural allergens (Thomas and Chua, 1993).

In this paper we describe the isolation of biologically active group 1 and 5 allergens from *Lolium perenne* and *Phleum pratense* by means of reverse phase chromatography on HPLC. In addition, this technique is suitable for the separation of group 5 allergens into 5a and 5b isoforms (Matthiesen and Lowenstein, 1991).

2. Material and methods

2.1. Extracts

The following lyophilized pollen extracts were obtained from Allergopharma, Reinbek, Germany: *Dactylis glomerata*, *Festuca pratensis*, *Holcus lanatus*, *Poa pratensis*, *Lolium perenne*, *Phleum pratense*.

The protein content of extracts was measured by a modified Bradford method on microtiter plates as described by Vieths et al. (1992).

2.2. Monoclonal antibodies

MAbs were produced after immunization with Timothy grass pollen extract (Müller et al., 1988) or an enriched Lol p 1.

2.3. Purification of group 5 allergens

Phl p 5 and Lol p 5 were isolated by affinity chromatography on mAb 1D11 coupled Sepharose 4B-CL and characterized as described earlier (Jung et al., 1989).

2.4. Separation of extracts by HPLC

For analytical studies the crude extracts or affinity purified group 5 allergens (about 50–100 µg protein) were separated by reverse phase HPLC (Shimadzu) using a Vydac C4 214 TP 52 column (2.1 mm × 250 mm). Preparative separations were performed with the ProRPC 5/2 equipment (Pharmacia, Uppsala) using the same elution conditions. An aqueous solution of 0.1% trifluoroacetic acid (TFA) was used as eluent A and 0.1% TFA in acetonitrile as eluent B. A linear gradient was applied for 30 min with flow rates of 0.5 ml and the absorbance of the eluent was measured at 220 and 280 nm. The eluted fractions were collected and lyophilized.

2.5. SDS-PAGE

SDS-PAGE analysis of proteins was performed under reducing conditions with ready-to-use Pharmacia Excel polyacrylamide gradient gels (8–18%) according to the instructions of the manufacturer (Pharmacia, Uppsala).

2.6. Dot blotting and immunodetection

Each fraction (about 0.5 µg/µl) was applied to a nitrocellulose membrane. After drying of the spots, the NC membrane was blocked for 30 min in a solution of PBS, 0.5% Tween 20 (TPBS). All further washing steps were done in TPBS. The blots were incubated overnight with hybridoma supernatants of mAb 9C12 or 1D11. After washing, bound mouse antibody was detected with biotinylated anti-mouse Ig (Boehringer Mannheim, diluted 1/1000 in TPBS) and with streptavidin-HRP (Boehringer Mannheim, diluted 1/2000 in TPBS). Then the blot was washed and developed using 4-chloro-1-naphthol (Serva).

For the detection of allergens the blots were incubated in patients' pooled serum diluted 1/20 in

washing buffer overnight. After washing, the NC membrane was incubated in mouse anti-human IgE (Immunotech, Hamburg, Germany) diluted 1/5000 and further developed as described above.

3. Results

Fig. 1 shows the separation of crude extracts of *Phleum pratense* and *Lolium perenne* grass pollen

extracts by HPLC gradient elution on a Vydac C4 column. The chromatograms of the various extracts were similar, but the relative proportions of proteins were very different. To identify allergenic components dot blotting was used to evaluate the fractions with regard to their ability to bind human IgE antibodies. IgE-binding components were found mainly in fractions with retention times of 18.5–26.5.

In order to locate and characterize the protein fractions of *Phleum pratense* and *Lolium perenne*

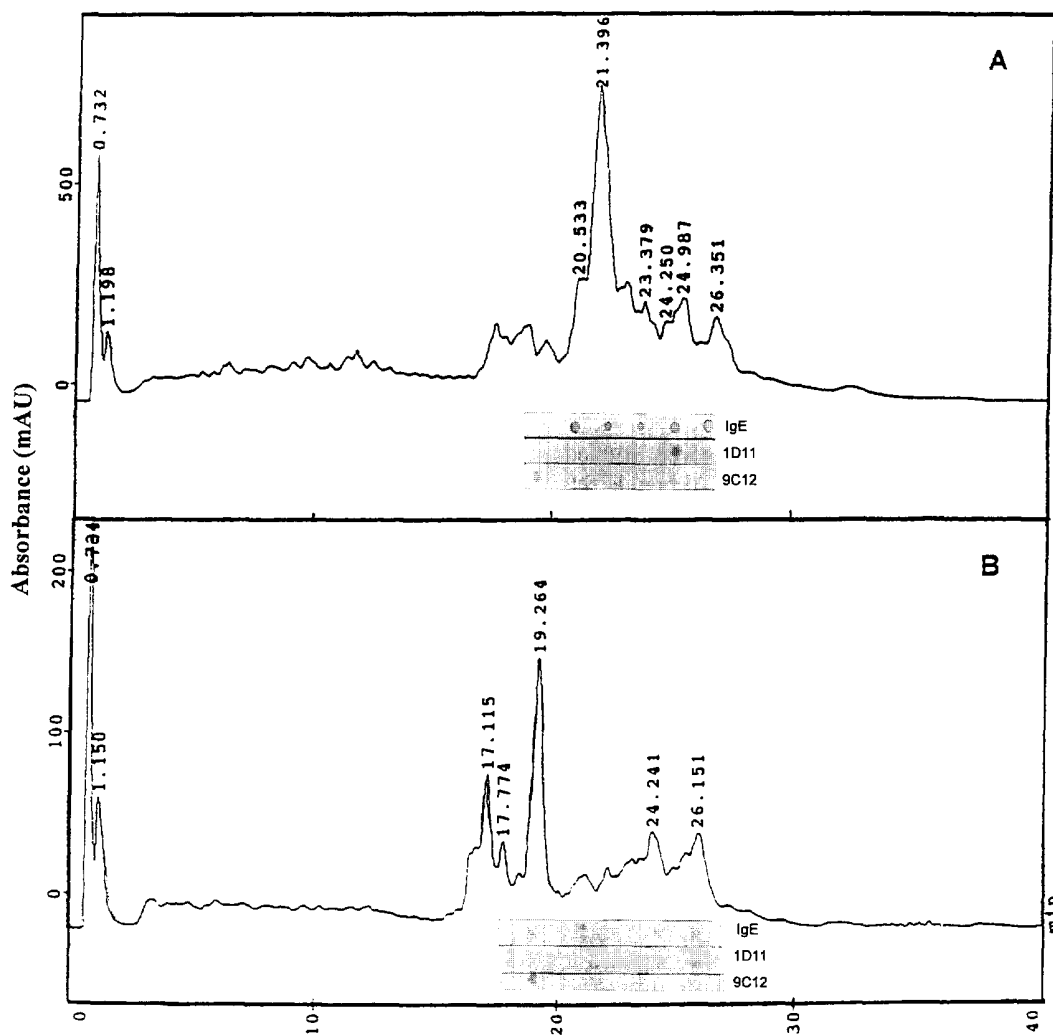


Fig. 1. Separation of whole pollen extract using reversed-phase chromatography. 50 μ g *Phleum pratense* (A) or 30 μ g *Lolium perenne* (B) were applied to a Vydac C4 214 TP 52 column. Gradient elution was performed with water/acetonitrile containing 0.1% TFA. Dot blot analysis of fractions are demonstrated beneath the chromatograms. The NC strips were incubated with patients' pooled serum (IgE), or with mAb 1D11 (anti-group 5), or with mAb 9C12 (anti-group 1).

representing the respective major allergens, dot blots were performed with group specific mAbs 9C12 (anti-group 1) and 1D11 (anti-group 5). The binding patterns of each fraction are indicated in Fig. 1. Group 1 allergens were identified in fractions with retention times of 19.1–20.5 min and group 5 in fractions with retention times of 24.3–26.3 min. After rechromatography of the main fraction of *Lolium perenne* (retention time of 19–20 min) a single peak was eluted at 19.3 min (Fig. 23). SDS-PAGE showed that this fraction had a molecular weight of 33 kDa, which corresponds to Lol p 1.

Group 5 allergens of *Lolium perenne* and *Phleum pratense* pollen extracts were purified by affinity chromatography using mAb 1D11 (specific for group 5 (Jung et al., 1989)) and this material was then used to obtain further information about the HPLC gradient elution patterns on the Vydac C4 column (Fig. 3). The group 5 allergens were eluted in at least two separate peaks. Using SDS-PAGE the peaks from the Phl p 5 separation were characterized by silver staining and appeared as dark brown stained bands corresponding to components Phl p 5a and Phl p 5b with molecular weights of 33 kDa and 29 kDa, respectively.

Four additional grass pollen extracts were studied by HPLC gradient elution (Fig. 4). Although the

chromatograms appeared to be different, the mAb binding patterns to group 1 and 5 allergens were similar.

4. Discussion

In order to achieve a better understanding of the allergenicity of grass pollen, it is important to isolate and characterize individual allergens. The most important allergenic components, group 1 and group 5, have been characterized not only by biochemical and immunochemical methods, but also by molecular biological techniques (Perez et al., 1990; Griffith et al., 1991; Silvanovich et al., 1991; Singh et al., 1991; Vrtala et al., 1993). It has been demonstrated that pollen allergens of the same group derived from different grass species are immunochemically similar in terms of antigenicity (Singh and Knox, 1985), IgE-binding capacity (Van Ree et al., 1991) and primary structure (high amino acid sequence homology) (Klysner et al., 1992). To date, no immunochemical relationship has been identified between group 1 and 5 allergens. Recently single T cell clones have been described which exhibited cross-reactivity with group 1 and group 5 allergens (Mohapatra et al., 1994; Müller et al., 1996). The most

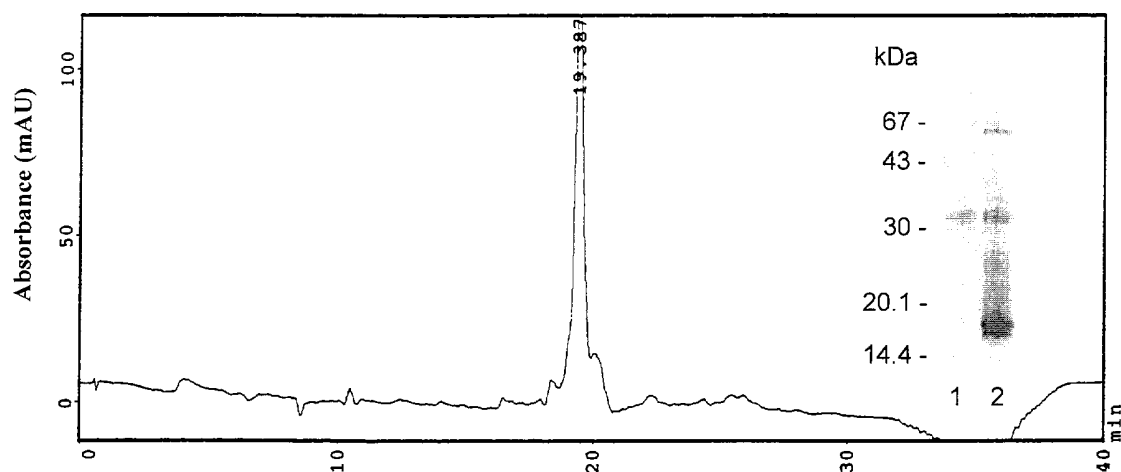


Fig. 2. Rechromatography of *Lolium perenne* fraction with retention time of 19–20 min (see Fig. 1B). Insert: SDS-PAGE analysis of the peak (lane 1); lane 2: *Lolium perenne* extract.

important prerequisite for these investigations were sources of highly purified allergens (>98%) in an active form. Using recombinant allergens the capability to stimulate the allergen-specific T cell clones was often less than that of the corresponding natural allergen (W.-D. Müller, personal communication).

The similarity in molecular weight of group 1 and 5 allergens has hindered their isolation in highly purified form for many years. Group 1 allergens are glycoproteins (Johnson and Marsh, 1965) with molecular weights of 33–34 kDa and group 5 allergens are pure proteins with molecular weights of 26–33 kDa (Petersen et al., 1991). In view of the

fact that group 1 molecules contain carbohydrates (about 5%) (Howlett and Clarke, 1981; Petersen et al., 1995) these allergens can be expected to possess more hydrophilic properties than the group 5 allergens. This difference may explain why reverse phase chromatography provides a suitable method for the separation of group 1 and group 5 allergens.

The chromatograms (Fig. 1) showed very different retention times for group 1 and group 5 containing fractions. Highly purified Lol p 1 could be isolated on a Vydac column after re-chromatography (Fig. 2). Similar results were obtained by Obispo et al. (1994) after separation of *Lolium perenne* extract

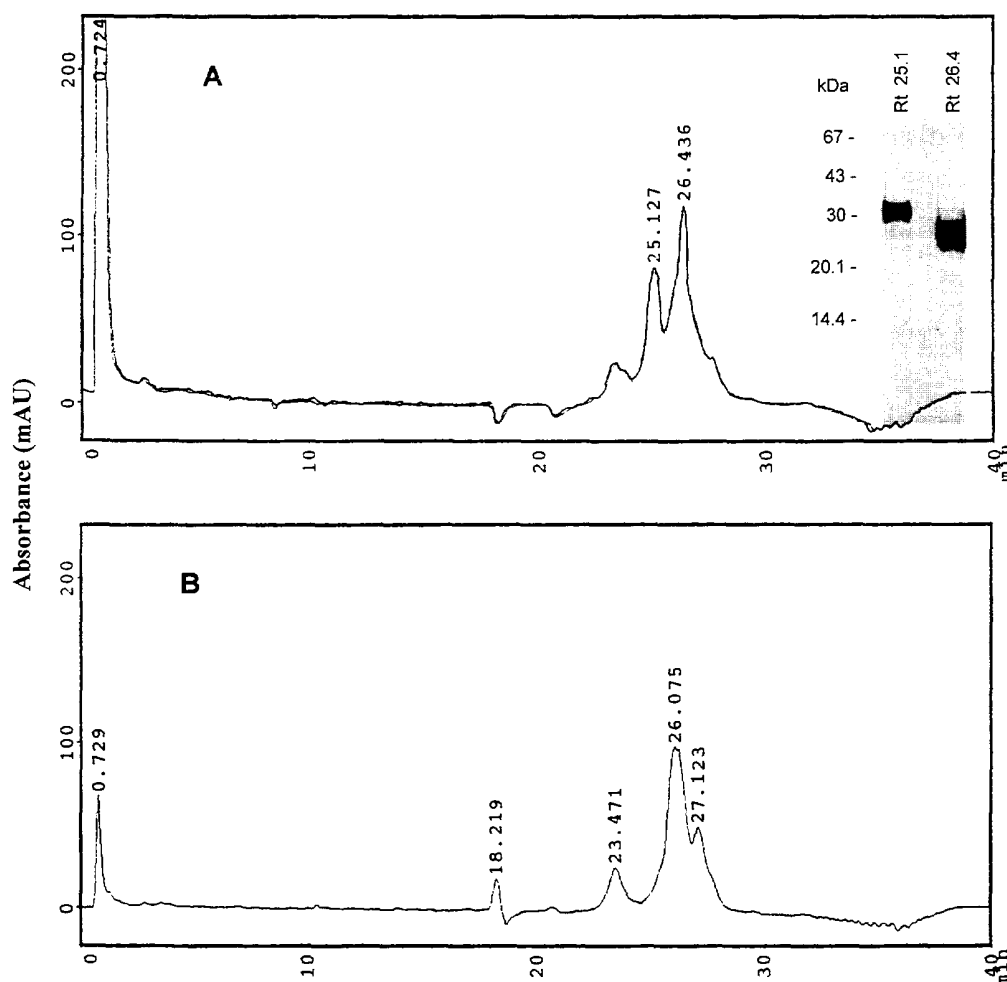


Fig. 3. Separation of Phl p 5 (A) and Lol p 5 (B) on RP-C4 column. Both proteins were isolated by affinity chromatography with mAb 1D11 (Jung et al., 1989). Insert, SDS-PAGE analysis of peaks with retention time of 25.1 (Phl p 5a) and 26.4 (Phl p 5b).

on HPLC. Although the group 1 and 5 allergens have very similar molecular weights as determined by SDS-PAGE, they could be distinguished from each other by colour differences after silver staining; group 5 allergens stained dark brown, whereas group 1 allergens stained brown-orange.

Our experiments show unequivocally that reverse phase chromatography is a suitable method for the separation of group 1 and group 5 allergens from crude pollen extracts in an immunoreactive form. The yield of the purified allergens was approximately 15–20% of the protein applied. The degree of purity of Lol p 1 and Phl p 5 was analysed by comparing each allergen preparation in a two site mAb assay for group 1 and group 5 (Fahlbusch et al., 1994). No evidence of cross contamination between the two allergen groups was found (data not shown). The affinity purified Phl p 5 contained insignificant amounts of group 1 allergen (< 0.5%).

Phl p 5 purified by affinity chromatography was separated on a Vydac column into Phl p 5a and 5b. The proteins have molecular weights of 33 kDa and 29 kDa respectively (Matthiesen and Lowenstein, 1991). This result suggests that our affinity purified group 5 is a mixture of the two subgroups 5a and 5b. This is an important prerequisite for the application of mAb 1D11 for the quantification of group 5 allergens in grass pollen extracts (Fahlbusch et al., 1993). Petersen et al. (1992) demonstrated, by means of 2D electrophoresis and immunoblotting, that each allergen consists of four isoallergens. The primary structures and the amino acid compositions of the eight proteins were determined and no differences were found in the 20 amino-terminal amino acid residues of proteins having the same molecular weight (Petersen et al., 1994).

A comparison of the elution profiles of different grass extracts demonstrates that group 1 and 5 aller-

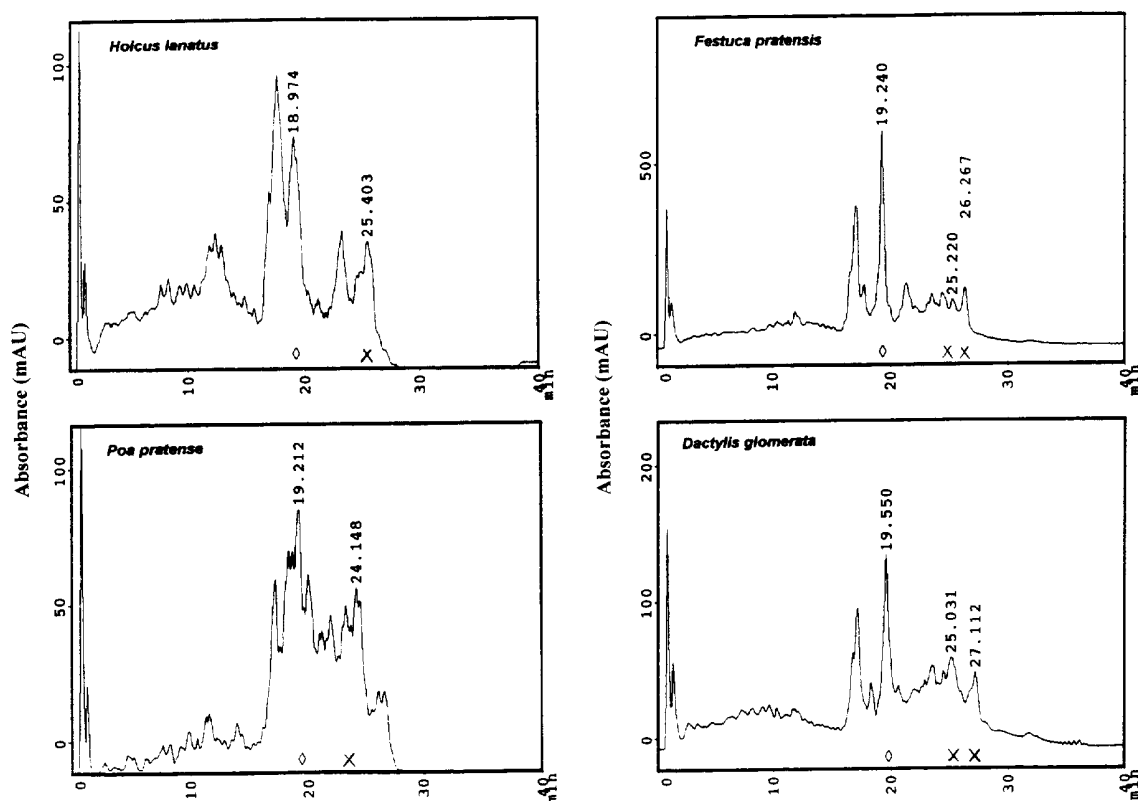


Fig. 4. Elution profile of four grass pollen extracts on RP-C4 column. Positive dot blot patterns with 1D11 (X) and 9C12 (◇) are indicated on each profile.

gens are indeed present in fractions with similar retention times (Fig. 4), but the relative amounts of the proteins are very different. *Dactylis glomerata*, *Festuca pratensis* and *Lolium perenne* contain appreciably higher concentrations of group 1 allergen by comparison with *Holcus lanatus*, *Poa pratensis* and *Phleum pratense*. These differences were also found by a two-site binding assay with mAbs to group 1 (unpublished data). A similar variability in the content of group 5 allergen was noted in several grasses when a two-site binding assay was used (Fahlbusch et al., 1994). This suggests that the reverse phase HPLC technique is also suitable for estimating the amounts of the different allergens in the extracts.

The application of reverse phase HPLC for the separation of allergens from crude extracts has several advantages. The allergens can be isolated from crude extracts by preparative HPLC using a ProRPC column much faster than by classical gel filtration methods, and the isolated proteins represent excellent tools for studying the epitopes recognized by T cells and B cells (Müller et al., 1994).

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