

Rapid and efficient purification of *Phleum pratense* major allergens Phl p 1 and group Phl p 2/3 using a two-step procedure

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Abstract

The standardization of natural allergenic extracts and the characterization of recombinant allergens ensures a continuing requirement for highly purified natural allergens. The extraction and purification methods have to be reproducible and also preserve the biological and immunological activity of the allergen. A simple two-step purification system has been established in order to provide milligram amounts of purified natural Phl p 1 and Phl p 2/3. Both major allergens were separated from other proteins of timothy grass pollen extract in one step by hydrophobic interaction chromatography (HIC) under mild conditions. The allergens elute in the flow-through fraction while the rest of the proteins remain bound to the column. The very different molecular weights of Phl p 1 and Phl p 2/3 permitted separation of the allergens by a second step using gel filtration. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Purified major allergens from pollen extracts are an essential requirement for many applications in research. It has been proposed that recombinant grass allergens, which can be expressed in *Escherichia coli*, can be used for diagnostic and therapeutic purposes (Laffer et al., 1994a; Petersen et al., 1995a; Vrtala et al., 1996). In order to compare recombinant allergens with their natural counterparts in respect of their allergenic potential, it is necessary to use sensitive methods such as the RAST inhibition assay or T-cell proliferation assays (Scheiner and Kraft, 1995).

Both assays are based on the availability of purified natural allergens.

The quantification of individual allergens in the standardization of natural allergen extracts is a further example of an application for purified allergens. Since recombinant allergens expressed in bacteria lack postranslational modification, such as glycosylation or phosphorylation, the use of natural allergens is preferable.

The applications mentioned above require, on the one hand, highly purified natural allergens and, on the other hand, adequate amounts of protein. Moreover the structure and functional activity of the natural allergen should be retained. Methods described thus far, such as affinity chromatography using mAbs (Boutin et al., 1997) or reversed-phase

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HPLC (Fahlbusch et al., 1996; Müller et al., 1996), use either harsh conditions during the purification or are limited in their capacity.

It has been considered impossible to separate the most important allergens of *Phleum pratense*, Phl p 1 and Phl p 5, with conventional chromatographic methods (Fahlbusch et al., 1996). In this paper, we describe the purification of the major allergen Phl p 1 and group Phl p 2/3 in a two-step procedure under mild conditions. The method is suitable for scaling up and can therefore be used for preparative purposes.

2. Material and methods

2.1. Timothy grass pollen extracts

Fours grams of timothy grass pollen (Allergon, Ångelholm, Sweden), previously defatted with petroleum ether followed by air drying, were suspended in 20 ml of 0.02 M Tris/HCl (pH 8.0), 1 mM EDTA and incubated under agitation at room temperature for 30 min. After centrifugation for 5 min at $22,000 \times g$, the supernatant was used directly for chromatography.

2.2. Purification of Phl p 1 and group Phl p 2/3

In order to perform hydrophobic interaction chromatography (HIC), ammonium sulphate was added to the extract to provide starting buffer conditions (1 M $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris/HCl, 1 mM EDTA, pH 8.0). Alternatively, lyophilized timothy grass pollen extract (Allergopharma, Reinbek, Germany) was reconstituted in starting buffer to a protein concentration of 20 mg/ml. After 0.45 μm filtration (Sartorius, Göttingen, Germany), the protein solution was applied to a C26/40 column (Pharmacia, Freiburg, Germany) packed with 70 ml Phenyl Sepharose High Performance (Pharmacia) and eluted at a flow rate of 5 ml/min. Bound proteins were eluted with distilled water. The volume of the flow-through fraction was reduced to about 1/10 with an ultrafiltration device Centriprep-10 (Millipore, Eschborn, Germany). Further purification of the concentrated flow-through fraction was achieved using 1 l Superdex 75 prep grade (Pharmacia) packed in a XK 50/60 column

(Pharmacia) at a flow rate of 5 ml/min with 50 mmol $(\text{NH}_4)\text{HCO}_3$. Three fractions were collected and either stored at -20°C or lyophilized.

2.3. Protein determination

The protein concentrations were determined using the Bio-Rad DC protein assay (Bio-Rad, Munich, Germany) according to the manufacturer's instructions. Bovine serum albumin was used as a protein standard.

2.4. SDS-PAGE and immunoblots

Proteins were separated by SDS-PAGE (13.5% T, 4% C; size $120 \times 80 \times 0.8 \text{ mm}^3$) using the buffer system of Laemmli (1970). After electrophoresis, proteins were either stained or subsequently transferred onto a supported nitrocellulose membrane (Sartorius) by semidry blotting for 30 min at 2 mA/cm².

2.5. Native isoelectric focusing

Native analysis of proteins according to isoelectric points was performed using agarose-based gels containing 4% (v/v) Servalyt (Boehringer Ingelheim Bioproducts, Heidelberg, Germany) with a pH-gradient ranging from 4.0 to 9.0.

2.6. Protein staining and immunodetection

Separated proteins were stained by coomassie blue-based Roti-Blue (Roth, Karlsruhe, Germany) according to the manufacturer's instructions.

Identification of individual allergens was performed using mAbs specific for Phl p 4 (5H1, 3C4C2; Fahlbusch et al., 1998), Lol p 1 (9C12; Fahlbusch et al., 1996), Phl p 5 (1D11C8; Müller et al., 1988; Fahlbusch et al., 1993), Phl p 6 (Bo 12, kindly provided by Dr. W.-M. Becker, Borstel, Germany) and recombinant human anti-Phl p 2 Fab (kindly provided by Prof. R. Valenta, Vienna, Austria). Prior to immunodetection, membranes were blocked by treatment for 30 min with Tris buffered saline (TBS, pH 7.4) supplemented with 0.05% (v/v) Tween 20. Incubation with 1/10 diluted patients' pooled serum or mAbs was carried out overnight. After washing,

bound antibodies were detected with alkaline phosphatase-conjugated monoclonal mouse anti-human IgE (Allergopharma) diluted 1/2000 or alkaline phosphatase-conjugated goat anti-mouse IgG/M diluted 1/10,000 (Dianova, Hamburg, Germany). The binding patterns were visualised by a substrate solution consisting of NBT/BCIP (Gibco-BRL, Gaithersburg, USA) in 100 mM Tris/HCl buffered saline solution (pH 9.5).

3. Results

Elution profiles of the chromatographic steps and SDS-PAGE analysis of relevant fractions are displayed in Fig. 1. Initial purification of the start

material using Phenyl Sepharose resulted in a brownish coloured flow-through fraction (Fig. 1A) containing three proteins with apparent molecular weights of approximately 55, 33/35 and 12 kDa (Fig. 1C, lane 1). Following size exclusion chromatography (Fig. 1B), proteins of the flow-through fraction were completely separated (Fig. 1C, lanes 3–5). Storage of fraction 3, even at 4°C, led to a decrease in the amount of the 55 kDa protein and the appearance of smaller proteins, possibly reflecting protease activity in this fraction. The reddish colour of fraction 5 suggested that the small protein retained tightly adsorbed pigments (Berrens et al., 1998). The peak at the end of the elution volume contained yellow-brown-coloured free pigments.

Immunological investigations on the five representative fractions were performed to identify the

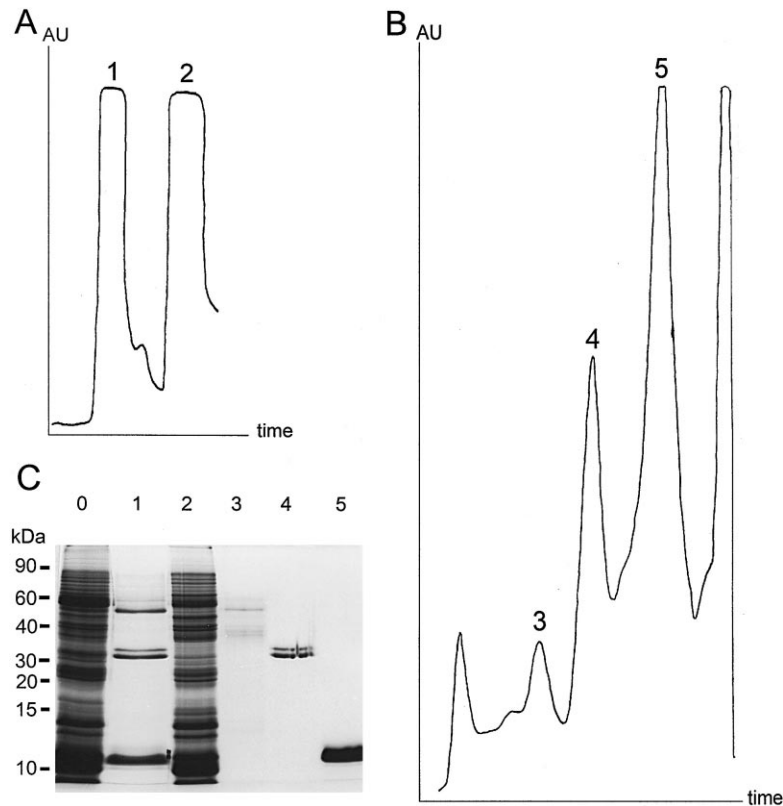


Fig. 1. Elution profiles and SDS-PAGE analysis of relevant fractions using a two-step purification scheme for separation of Phl p 1 and Phl p 2/3 from timothy grass pollen extract. (A) Timothy grass pollen extract was applied to a Phenyl Sepharose high performance gel. The flow-through fraction (1) and eluted proteins (2) were collected. (B) The flow-through was subsequently purified on Superdex 75. (C) SDS-PAGE analysis of timothy grass pollen extract (0) and fractions as in A and B indicated (1–5).

proteins and to analyse the efficiency as well as the completeness of the purification.

Each of the purified proteins was clearly IgE-reactive as demonstrated with a pool of serum derived from subjects allergic to timothy grass pollen (Fig. 2A). Although the 55 kDa protein exhibited a similar size compared with Phl p 4, no reaction was detected with Phl p 4 specific mAbs (Fig. 1B). Moreover, in contrast to the unidentified 55 kDa protein, Phl p 4 was completely bound to the HIC medium. Using the group 1 specific mAb 9C12 the purified 33/35 kDa double band could be identified as Phl p 1 (Fig. 2C).

In the case of *P. pratense*, group 1 isoallergens appear in at least two different molecular sizes (Petersen et al., 1993). The faint immunostaining of Phl p 1 in the grass pollen extract (Fig. 2C, lane 0) compared with the purified allergen (Fig. 2C, lanes 1 and 4) was possibly caused by the low amount of Phl p 1 present in the extract or by partial degradation. The purified 12 kDa protein was clearly identifiable by binding of a recombinant Fab fragment specific for Phl p 2 (Fig. 2D). Since Phl p 2 and Phl p 3 presumably share similar attributes including molecular weight and amino acid sequence (Dolecek et al.,

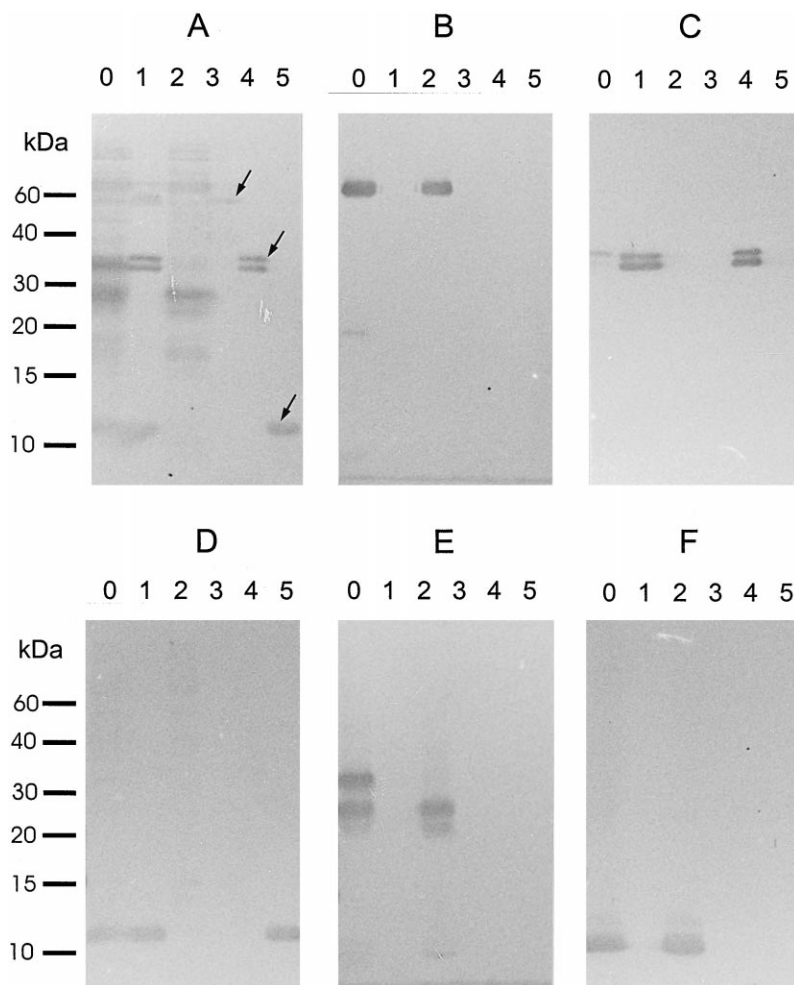


Fig. 2. Immunological analysis of relevant fractions (see Fig. 1). Six identical blots derived from reducing SDS-PAGE carrying the same samples in lanes 0 to 5 as in Fig. 1 were treated with (A) patients' pooled serum ($n = 5$); (B) group 4 specific monoclonal antibodies; (C) group 1 specific monoclonal antibody; (D) recombinant anti Phl p 2 Fab; (E) group 5 specific monoclonal antibody and (F) group 6 specific monoclonal antibody. Arrows in (A) indicate the position of the purified IgE-reactive proteins.

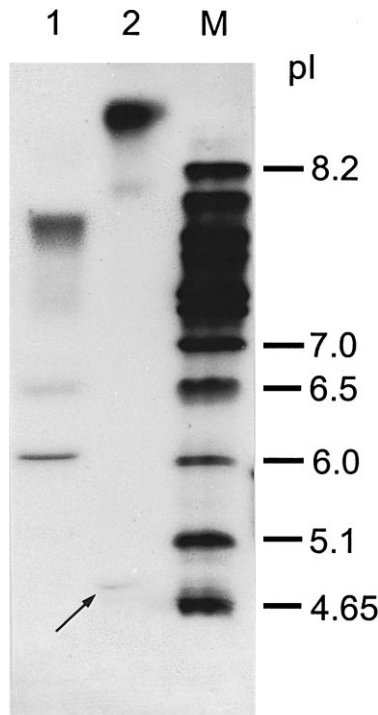


Fig. 3. Native isoelectric focusing of purified allergens. Analysis of isoforms of (1) Phl p 1 and (2) Phl p 2/3 was performed by coomassie staining. Lane M contains IEF standard ranging from pI 4.65 to pI 9.6. The arrow indicates the location of Phl p 2.

1993), it was considered probable that this fraction contained both Phl p 2 and Phl p 3.

HIC affects almost complete separation of Phl p 1 and group Phl p 2/3 from other proteins of the timothy grass pollen extract. The yield of purified allergens was approximately 0.5% and 1%, respectively, of the protein applied. Other IgE-reactive components and proteins, with the exception of the unidentified 55 kDa allergen, remained quantitatively attached to the Phenyl Sepharose. Elution of bound proteins with distilled water resulted in recovery of Phl p 5b, Phl p 6 and Phl p 4. In the case of group 5 allergens *P. pratense* contains two subgroups of isoforms, designated as Phl p 5a and Phl p 5b, which differ in their primary structure (Gelhar et al., 1997) and molecular size. Interestingly, whilst Phl p 5b was eluted using distilled water, Phl p 5a was not recovered (Fig. 2E and F). It is probable that Phl p 5a binds too tightly to Phenyl Sepharose to be eluted under these conditions.

A subsequent gel filtration step served to effect efficient separation of Phl p 1 and Phl p 2/3 as well as enhancing purity and desalting. There was no Phl p 2/3 detectable in the Phl p 1 fraction and vice versa (Fig. 2C and D). Even minor amounts of possible impurities, such as Phl p 5 or Phl p 6, could not be detected in the Phl p 1 or Phl p 2/3 fractions (Fig. 2E and F).

Further characterization of purified proteins was performed using native isoelectric focusing (Fig. 3). Both the Phl p 1 and group Phl p 2/3 fractions were composed of isoforms ranging from pH 6.1 to 7.9 and pH 8.1 to 9.0, respectively. A small portion of fraction 5 was located around pH 4.8 (Fig. 3, lane 2). In the case of the 12 kDa allergens of *Lolium perenne*, Ansari et al. (1987; 1989a; b) characterized two distinctive forms with similar amino acid sequences (Sidoli et al., 1993), but extremely different isoelectric points. These two forms were designated as Lol p 2 and Lol p 3. By analogy with these results, the same seems to be true for acidic Phl p 2 and basic Phl p 3.

A summary of the results and a schematic overview of the purification steps is presented in Fig. 4.

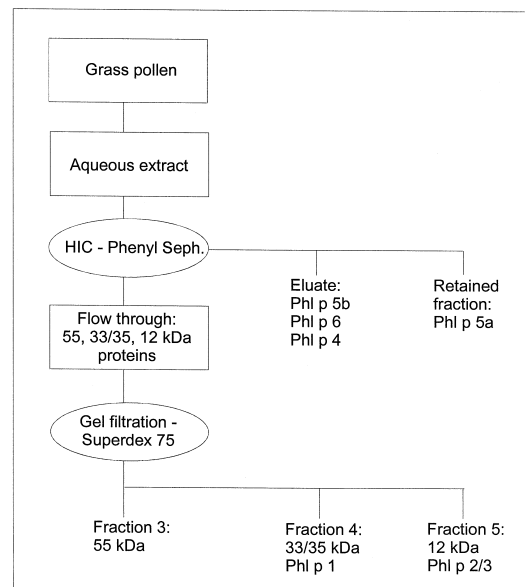


Fig. 4. Schematic overview of the purification steps and summary of the results.

4. Discussion

The most important allergens of *P. pratense*, group 1 and 5 allergens (Matthiesen and Lowenstein, 1991), share similar physicochemical parameters such as molecular weight and isoelectric point. For this reason, they are difficult to separate by ion exchange chromatography or gel filtration. Other methods, such as affinity chromatography and reversed phase high-performance chromatography, are disadvantageous as far as maintenance of biological activity or purification of large amounts of allergens are concerned.

Phl p 1 and Phl p 5 clearly differ regarding their chromatographic properties using HIC. This alternative non-denaturing method completely separates both major allergens. Moreover, Phl p 2/3 and Phl p 6, which show similar sequences to Phl p 1 and Phl p 5, respectively (Ansari et al., 1989a; Laffer et al., 1994b; Petersen et al., 1995b), are separated in the same way. This result indicates differences between the major allergens Phl p 1/Phl p 2/3 and Phl p 5/Phl p 6 concerning their hydrophobicity. Therefore, the use of HIC offers a useful supplementary method for the characterization or classification of grass pollen allergens. The separation scheme described in this study represents a powerful tool for purification of milligram amounts of at least two major allergens of timothy grass pollen. Sufficient pure protein can be prepared in order to compare natural allergens with their recombinant counterparts and thereby account for the differences in respect of IgE reactivity (Laffer et al., 1994b). Since natural Phl p 1 is glycosylated, interest turns to the possible IgE binding capacity of carbohydrate moieties. Various natural isoforms of Phl p 1 have been recognized (Petersen et al., 1993) and their structural micro-heterogeneity may give rise to different allergenicities.

The successful recombinant bacterial expression of insoluble proteins, such as Phl p 1 (Vrtala et al., 1996), requires denaturation and subsequent refolding in order to obtain the correct conformational structure (Marston, 1986), which is especially important for the activity of allergens. For example, Soldatova et al. (1998) demonstrated that expression of bee venom hyaluronidase in bacteria resulted in a lower specific enzymatic activity and IgE binding

capacity than that of the natural protein, properties attributable to inappropriate folding and disulphide bonding. Consequently, when estimating the IgE reactive fraction of a recombinant allergen preparation, it is advisable to make use of the purified natural counterpart.

The major groups of grass allergens from different species share not only primary structure homology (Dolecek et al., 1993; Vrtala et al., 1996) but also immunological cross-reactivity (Fahlbusch et al., 1998; Niederberger et al., 1998). Based on many examples of similarity between *L. perenne* and *P. pratense* allergens, it was assumed that the findings of Ansari et al. (1989a) concerning group 2/3 allergens would apply also to timothy grass pollen. The data presented in this study correspond exactly to those reported for Lol p 3. Since Lol p 2 and Lol p 3 differ in respect of their isoelectric points with values of 4.5–5.0 and 9–9.4, respectively (Ansari et al., 1989a,b), the same may be assumed for Phl p 2 and Phl p 3.

In the case of group Phl p 2/3, additional procedures have to be undertaken to separate distinctive isoforms making use of different isoelectric points. Interestingly, under the conditions used in the present study, Phl p 3 is clearly more abundant than Phl p 2. For that reason, it is important to consider the possibility that Phl p 3 is more important in terms of IgE-binding frequency and capacity than Phl p 2, although in the case of ryegrass, both allergens seem to be crossreactive at the IgE-level (Ansari et al., 1987). Taking the relatively low sequence identity of 59% between Lol p 2 and Lol p 3 into account (Ansari et al., 1989a), further differences may exist in respect of their reactivities at the T-cell level. This may have some influence on the choice of composition of future recombinant allergen-based therapeutics with respect to T-cell epitopes. The successful separation of the allergens will enable interest to be focused on the significance of individual T-cell reactivities and other aspects such as IgE cross-reactivity between allergen group Phl p 2/3 and Phl p 1 (Roberts et al., 1992).

Apart from the efficient purification of at least two major allergens of *P. pratense*, the protein fraction eluted from Phenyl Sepharose may also prove to be useful. This fraction represents an allergen extract depleted of Phl p 1, Phl p 2/3 and Phl p

5a. Since other major allergens, such as Phl p 5b, Phl p 4 and Phl p 6, are recovered without loss, this allergen solution may serve as a negative or positive control in a variety of experiments. It may also serve as a starting point for the purification of other allergens.

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