

# Size exclusion chromatography as a tool for quality control of recombinant allergens and hypoallergenic variants

Bernhard Weber\*, Holger Slamal, Roland Suck

*Allergopharma Joachim Ganzer KG Hermann-Körner-Straße 52, D-21465 Reinbek, Germany*

## Abstract

Proteins or glycoproteins bearing epitopes for human IgE antibodies are designated as allergens causing type I allergic diseases. In this study, recombinant allergens were compared with their natural counterparts either as part of extracts or as purified molecules with respect to several biochemical and immunological properties.

Natural and recombinant Bet v 1 and Phl p 1, major allergens of birch pollen extracts and *Phleum pratense* pollen extracts, were analyzed by SDS-PAGE, immunoblotting, EAST inhibition and size exclusion chromatography (SEC).

Differences of IgE-binding capacities between recombinant Bet v 1 as well as recombinant Phl p 1 variants were detected by EAST inhibition. These results were confirmed by size exclusion chromatography in that the recombinant proteins showed differences of their elution volumes being equivalent to the natural molecules only with the more active recombinant form. In contrast, SDS-PAGE and immunoblot analysis resulted in divergent characteristics, as either migrations of the variants were similar or no differences of IgE binding were detectable.

In conclusion, size exclusion chromatography is the method of choice for quality control of well characterized recombinant allergens, comprising control of purity, protein content and conformation. © 2003 Elsevier Science B.V. All rights reserved.

**Keywords:** Size exclusion chromatography; Allergen; SDS-PAGE

## 1. Introduction

Allergens causing IgE-dependent type I allergy [1] are characterized as proteins or glycoproteins bearing epitopes for human IgE. Extracts produced from raw material such

\* Corresponding author. Tel.: +49-40-727-65-166.

E-mail address: [bernhard.weber@allergopharma.de](mailto:bernhard.weber@allergopharma.de) (B. Weber).

as house dust mite or pollen are used for the production of preparations for diagnosis and therapy of allergic diseases. Several techniques have been used in order to achieve standardisation of allergen extracts, which in general can be mixtures of more than 100 proteins. Size exclusion chromatography (SEC) was introduced as one method for quality control of allergen extracts as soon as high performance matrices were available, leading to chromatograms characteristic for the extract to be analyzed [2]. Furthermore, SEC was applied in order to separate low molecular weight molecules, including non-proteinaceous material from the extracts.

Since 1990, molecular biology has been established in allergology, and to date, primary structures of more than 700 allergens have been discovered by cloning techniques [3–5]. The next step, production of allergens by recombinant techniques, has been developed more or less in parallel, and recombinant allergens were used for several in vivo and in vitro investigations [6,7]. These studies demonstrate that recombinant allergens are as effective and safe as their natural counterparts. Commercial production of preparations for therapeutic or diagnostic use may take advantage of unique features characteristic for recombinant proteins, such as purity and homogeneity, providing batch-to-batch consistencies not achievable with crude allergen extracts. Aiming at a strong simplification of standardisation procedures required for crude allergen extracts as described, i.e. in the European Pharmacopoeia, accompanied by significantly higher quality levels—i.e. with respect to 50–200% limits for biological activity or even for quantification of allergens in crude extracts—recombinant allergens could be standardised by protein quantification only.

In general, two variants of recombinant allergens can be produced: one kind to be used as a diagnostic tool for in vivo or in vitro applications representing a true copy of its natural counterpart with respect to primary structure, IgE-binding capacity and activity measured by several methods such as histamine release, T cell stimulation or skin prick tests, and the other kind with significantly lower IgE-binding capacity to be used for therapy. The latter variant may be constructed by changes to the primary structure [8], but as IgE binding is strongly dependent on the conformation of the allergen, it can also be affected by the production methods for recombinant allergens with exactly the same primary structures due to expression systems (e.g. prokaryotic or eukaryotic), posttranslational modifications (glycosylation) and purification schemes [9]. In addition, undirected changes occurring during up- and down-stream processing may influence the secondary structure cutting down the quality of the product. Therefore, recombinant allergens require a detailed characterization of structure and conformation.

In this study, variants of two recombinant allergens, a major allergen of timothy grass pollen (*Phleum pratense*) rPhl p 1 and a major allergen of birch pollen (*Betula verrucosa*) rBet v 1, were compared with natural molecules with respect to their IgE-binding capacities. Variants were produced with exactly the same primary structure in *E. coli*, using different purification schemes. IgE binding of the variants was found to be as high as in the natural allergen in one variant, and significantly lower in the other, indicating substantial differences of conformations. When analyzed by SDS-PAGE, the variants were not distinguishable with respect to their apparent molecular weights, but conformational changes could be effectively monitored by SEC. In conclusion, SEC may be used as the most reliable quality control method estimating purity, aggregations and conformational differences related to IgE-binding capacities.

## 2. Material and methods

### 2.1. Allergen extracts and purified natural allergens

Allergen extracts of timothy grass and birch pollen were produced by Allergopharma (Reinbek, Germany). For SEC and SDS-PAGE analysis, freeze dried extracts were reconstituted at 1 mg/ml in water.

The purification of Phl p 1 was performed according to Suck et al. [10]. Briefly, a timothy grass pollen extract was separated by hydrophobic interaction chromatography (HIC) using Phenyl Sepharose HP (Amersham biosciences, Freiburg, Germany). The flow-through fraction was subsequently concentrated with ultrafiltration devices Centriprep-10 (Cutoff: 10 kDa; Millipore, Eschborn, Germany). Proteins of the flow-through fraction were finally purified using gel filtration with Superdex 75 (Amersham biosciences).

Bet v 1 was purified out of crude birch pollen extracts by affinity chromatography based on a specific monoclonal antibody (mab). Immobilization of mab 2E10 (Allergopharma) was performed according to Harlow and Lane [11] as described [12]. Crude birch pollen extract (approximately 4 mg/run) was passed through the column, which contained approximately 2 ml Protein G-Sepharose prepared with 3 mg purified mab. After washing, bound Bet v 1 was eluted with 0.1 mol/l glycine buffer, pH 2.5, and immediately neutralized. Purified protein was dialysed with water and stored frozen dried at  $-20^{\circ}\text{C}$ .

### 2.2. Recombinant allergens

Recombinant Phl p 1 was produced in *E. coli* Origami pLysS (Novagen, Bad Soden, Germany) carrying a construct of a pProEx vector (Gibco BRL, La Jolla, USA) and the cDNA-sequence encoding Phl p 1 [13]. After expression, recombinant His-tagged Phl p 1 was purified from inclusion bodies, and after solubilisation in 6 mol/l guanidine chloride, chelating Sepharose (Amersham biosciences) loaded with nickel was used to renature the protein in phosphate buffer by a gradient over 10 column volumes. Elution of renatured rPhl p 1 was performed with 50 mM histidine. In order to cleave the His-tag, recombinant TEV protease (Gibco BRL) was added and incubated overnight at room temperature. Further purification of rPhl p 1 was achieved by hydrophobic interaction chromatography using Phenyl Sepharose and gel filtration on Superdex 75.

Recombinant Bet v 1 was expressed in *E. coli* BL 21 cells transformed with a pSE420 vector (Invitrogen, Karlsruhe, Germany) carrying the cDNA-sequence encoding Bet v 1 [14]. After cell lysis, soluble rBet v 1 was purified using anion exchange chromatography with Source 15 Q (Amersham biosciences), hydrophobic interaction chromatography with Phenyl Sepharose high performance (Amersham biosciences) and finally, gel filtration with either Tris-based (pH 8.0) or sodium hydroxide (pH 11.0) running buffers [15].

### 2.3. SDS-PAGE and Western blotting

Proteins were separated by SDS-PAGE (12% or 15% T, 4% C) with the buffer system of Laemmli [16]. Reducing and nonreducing sample buffers were the same except that

5 × reducing sample buffer contained 200 mmol/l DTT. After electrophoresis, proteins were either stained with Coomassie brilliant blue R-250 (Merck) or transferred onto a PVDF membrane (Sartorius, Göttingen, Germany) by semidry blotting for 30 min at 0.8 mA/cm<sup>2</sup>.

Immunological characterization of recombinant allergens was performed by using either specific mabs (2E10 for Bet v 1 and 9C12 for Phl p 1) or sera from subjects allergic to birch pollen. Prior to immunodetection, membranes were blocked by treatment for 30 min with Tris-buffered saline (TBS, pH 7.4) supplemented with 0.05% (w/v) Tween 20. Incubations with either 1:10 diluted patients' pooled serum or mabs were carried out overnight. After washing, bound antibodies were detected with alkaline phosphatase conjugated mouse monoclonal anti-human IgE (Allergopharma) diluted 1:2000 or with 1:10,000 diluted goat anti-mouse IgG/M with alkaline phosphatase (Dianova, Hamburg, Germany). Immunoblots were developed by chemiluminescence using CSPD as substrate and visualized by X-ray films.

#### 2.4. EAST inhibition

The relative IgE-reactivities were evaluated with an EAST inhibition assay as described [8]. Microtiter plates were coated with either nPhl p 1 or nBet v 1 (0.1 µg/well). Before loading to the microtiter plate, patients' sera (diluted 1:4) were preincubated with serial dilutions of the inhibitors rPhl p 1 MF, rPhl p 1 CF, nPhl p 1, rBet v 1 MF, rBet v 1 CF, nBet v 1 ( $1.0 \times 10^{-6}$  to  $5 \times 10^{-11}$  mol/l). Serum pools were composed of 10 individual sera derived from patients allergic to grass or birch pollen. IgE-binding capacity was estimated as that concentration required to reach 50% inhibition.

#### 2.5. Analytical size exclusion chromatography and fraction analysis

Analytical SEC was performed on two different chromatography systems using three different columns for comparison purposes. An Äkta explorer (Amersham biosciences) fitted with a HiPrep 16/60-column (16 × 600 mm, Amersham biosciences) filled with Sephacryl S-100 (particle size, 25–75 µm; cross-linked allyl dextran with *N,N'*-methylene bisacrylamide) or Superdex 75 (bead size, 11–15 µm; highly cross-linked porous agarose) were run with a flow rate of 1.0 ml/min. Running buffers were 25 mmol/l sodium phosphate buffer, 200 mmol/l NaCl, pH 7.5, or 154 mmol/l NaCl, respectively. The applied sample volume was 0.45% of the column volume.

An HPLC device (Thermo separations) was equipped with a TSK G 2000 SW column (7.5 × 600 mm, guard column 7.5 × 75 mm, particle size 10 µm; hydrophilic, rigid, spherical bonded silica; Toyo Soda, Tokyo, Japan), which was run at a flow rate of 1.0 ml/min using 0.5 mol/l sodium acetate, pH 6.8, as running buffer. The sample volume was 0.5% of the column volume. All running buffers were filtered through 0.2 µm filtration devices (Millipore, Göttingen, Germany) before use.

For immunological analysis of eluted proteins, fractions of 0.5 ml were collected and directly applied onto a nitrocellulose membrane, which was previously wetted with distilled water using a slot blot device 'convertible' (Gibco). Blocking and incubation with specific monoclonal antibodies was conducted as described above. The binding patterns were

visualized by a substrate solution consisting of NBT/BCIP (Gibco BRL, Gaithersburg, USA) in 100 mmol/l Tris/HCl-buffered saline solution (pH 9.5). In order to determine the amount of eluted protein, the immuno-stained membrane was digitally captured and densitometrically quantified using a 'Bio-doc system' (Biometra, Göttingen, Germany).

### 3. Results and discussion

In order to evaluate the potential of size exclusion chromatography for the purpose of quality control of allergenic recombinant proteins, two important allergens were investigated. In each case, four different preparations were analyzed by SDS-PAGE, IgE-binding properties and SEC elution profiles. A whole birch pollen extract, purified major birch pollen allergen Bet v 1 and two different recombinant preparations of this major allergen were investigated. The major difference concerning rBet v 1 variants was that either neutral or alkaline running buffers were used in identical downstream processes. Whole Timothy grass pollen extract, purified major allergen Phl p 1 and two different preparations of recombinant Phl p 1 were also examined. Differences detected between the rPhl p 1 preparations are very likely due to protein folding variants, as this molecule, in contrast to Bet v 1, contains cysteine residues and intramolecular disulphide bridges [13,17,18].

#### 3.1. SDS-PAGE of allergen preparations

##### 3.1.1. Birch pollen allergens

As shown in Fig. 1A, Bet v 1 appears as a prominent band in the birch pollen extract with an apparent molecular weight of approximately 17 kDa. Purified natural Bet v 1 gave exactly the same position, whereas slight differences of molecular weights were detected with rBet v 1 MF and rBet v 1 CF.

Binding of Bet v 1 specific mab 2E10 (Fig. 1B) was comparable with nBet v 1 in the extract and as purified molecule, and with rBet v 1 CF, but only a weak binding was detectable with rBet v 1 MF, indicating differences of binding sites between the recombinant variants. These findings were confirmed by IgE-binding capacities, which were not distinguishable between natural Bet v 1, either in the extract or as purified protein, and the recombinant CF variant. In contrast, IgE binding with the recombinant MF variant was not detectable (Fig. 1C).

In conclusion, differences of the recombinant Bet v 1 variants were not detectable by SDS-PAGE alone, as they do not affect the molecular weight as analyzed in this system. Changes of molecular structures of rBet v 1 MF became apparent when binding capacities for a monoclonal antibody and more pronounced for human IgE were analyzed by immunoblotting.

##### 3.1.2. *P. pratense* allergens

Phl p 1 is characterized by two features, which it does not share with Bet v 1 in that this molecule is a glycoprotein and contains cysteine residues, determining its behavior in separation by SDS-PAGE.

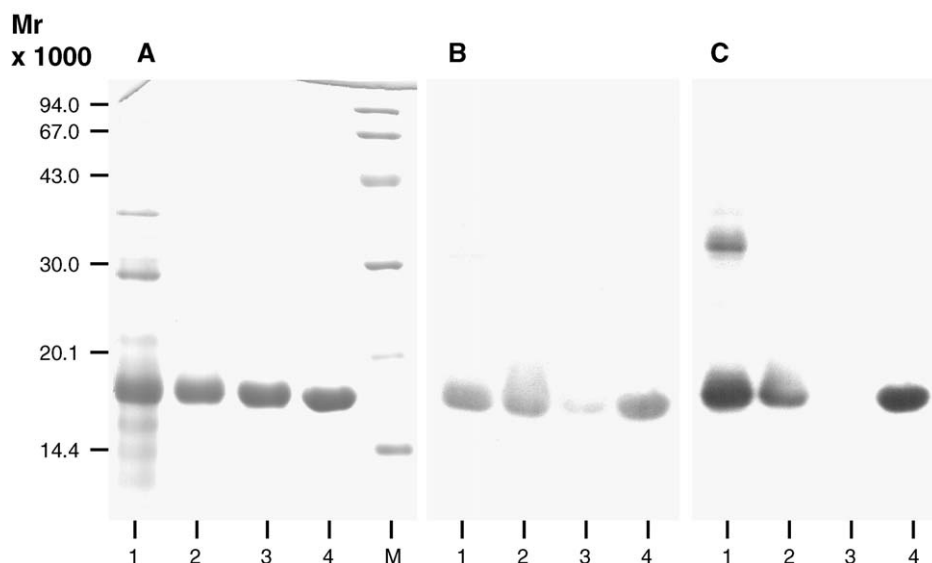


Fig. 1. Birch pollen extract, natural Bet v 1 and recombinant Bet v 1 variants analyzed by SDS-PAGE and immunoblot. Samples were separated under reducing conditions. (A) SDS-PAGE, protein staining (Coomassie). (B) Immunoblot, probed with Bet v 1 specific mab 2E10. (C) Immunoblot, probed with a pool of birch pollen allergic patients' sera (serum pool A). M: Molecular weight marker proteins; 1: birch pollen extract (30  $\mu$ g); 2: nBet v 1 (4  $\mu$ g); 3: rBet v 1 MF (4  $\mu$ g); 4: rBet v 1 CF (4  $\mu$ g).

The results of the analysis of *Phleum* allergens and Phl p 1 by SDS-PAGE under reducing conditions (Fig. 2A) were in accordance with their molecular properties in that purified nPhl p 1 gave a slightly higher molecular weight as that of the recombinant variants. rPhl p 1 MF and CF did not show any differences, neither in SDS-PAGE analysis nor in immunoblotting after probing with either mab 9C12 or human IgE (Fig. 2B,C).

When analyzed under nonreducing conditions, the protein pattern of the *P. pratense* extract as well as nPhl p 1 showed more diffuse bands, reflecting the carbohydrate moieties in several extract proteins and nPhl p 1 (Fig. 3A, lanes 1 and 2). The molecular weight of nPhl p 1 with approximately 30–28 kDa was lower than that of the reduced molecule with approximately 34 kDa. The recombinant variants appeared as predominantly one band for rPhl p 1 MF with a molecular weight of 28 kDa, whereas rPhl p 1 CF contained a main component with 25 kDa (Fig. 3A, lanes 3 and 4).

Immunoblot analysis, either with a Phl p 1 specific monoclonal antibody (Fig. 3B) or with a serum pool of human allergic patients' sera (Fig. 3C), did not give detectable differences between natural Phl p 1 and its recombinant variants, indicating that the different molecular weights of the recombinant variants do not affect their binding capacities, neither when compared with nPhl p 1 nor one variant with the other.

In conclusion, rPhl p 1 variants were distinguishable by SDS-PAGE when analyzed under nonreducing conditions in contrast to their reduced forms. Immunoblot analysis did not result in corresponding differences of binding capacities, as it was shown for the rBet v 1 variants.

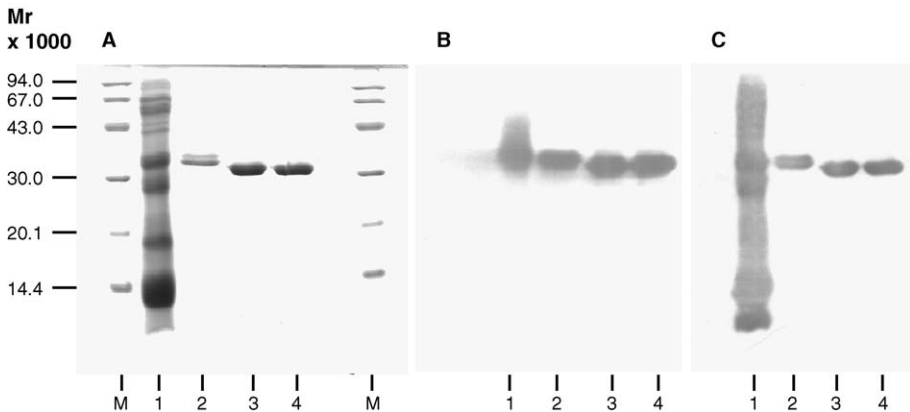


Fig. 2. *P. pratense* pollen extract, nPhl p 1 and recombinant Phl p 1 variants analyzed under reducing conditions by SDS-PAGE and immunoblot. (A) SDS-PAGE, protein staining (Coomassie). (B) Immunoblot, probed with Phl p 1 specific mab 9C12. (C) Immunoblot, probed with a pool of *P. pratense* pollen allergic patients' sera (serum pool B). M: Molecular weight marker proteins; 1: *P. pratense* pollen extract (30 µg); 2: nPhl p 1 (4 µg); 3: rPhl p 1 MF (4 µg); 4: rPhl p 1 CF (4 µg).

### 3.2. Comparison of IgE-binding capacities by using EAST inhibition

In the case of rBet v 1, the results obtained by SDS-PAGE were contrary to the immunoblot results in that rBet v 1 variants were not distinguishable by SDS-PAGE. In contrast, the rPhl p 1 variants appeared as two forms with respect to their molecular weights in SDS-PAGE but were not distinguishable by immunoblot. In order to detect also

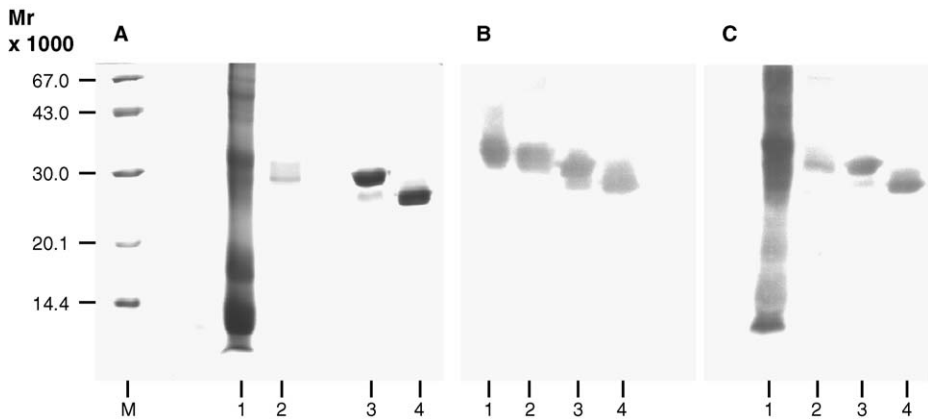


Fig. 3. *P. pratense* pollen extract, nPhl p 1 and recombinant Phl p 1 variants analyzed under nonreducing conditions by SDS-PAGE and immunoblot. (A) SDS-PAGE, protein staining (Coomassie). (B) Immunoblot, probed with Phl p 1 specific mab 9C12. (C) Immunoblot, probed with a pool of *P. pratense* pollen allergic patients' sera (serum pool B). M: Molecular weight marker proteins; 1: *P. pratense* pollen extract (30 µg); 2: nPhl p 1 (4 µg); 3: rPhl p 1 MF (4 µg); 4: rPhl p 1 CF (4 µg).

minor differences of IgE-binding capacities, especially in rPhl p 1 variants, an EAST inhibition assay was performed.

As shown in Fig. 4A, rPhl p 1 variants CF and MF as inhibitors of IgE binding were different with respect to the natural molecule in that their inhibition curves did not reach 100% inhibition. This might be due to the lack of carbohydrate moieties and in consequence to the lack of inhibition capacity for IgE antibodies binding with carbohydrate structures [19–21]. Furthermore, differences of IgE-binding capacities became apparent comparing the 50% inhibition values, as the inhibitory potency of rPhl p 1 CF was as effective as nPhl p 1, whereas an almost 10-fold concentration of rPhl p 1 MF was necessary to reach 50% inhibition. These results were contrary to the immunoblot results, demonstrating the limits of immunoblot analysis in quantitative aspects.

In contrast, the inhibition curves obtained with nBet v 1 and its recombinant variants confirmed the immunoblot analysis in that rBet v 1 CF functioned as an inhibitor, which was not distinguishable from nBet v 1, and rBet v 1 MF showed a very poor inhibitory potency in that an almost 100-fold concentration relative to that of nBet v 1 was required for 50% inhibition (Fig. 4B).

In conclusion, the determination of IgE-binding capacities of recombinant variants provided results, which were more reliable than those obtained by immunoblot analysis. In consequence, it seems to be necessary to apply this method in addition to SDS-PAGE and immunoblots in order to judge the quality of recombinant allergens correctly with respect to IgE-binding capacity.

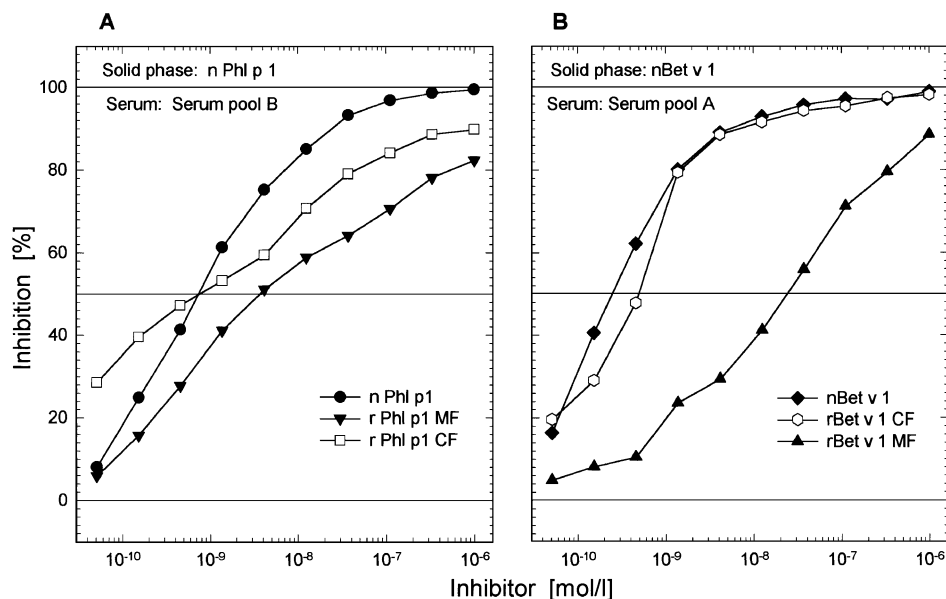


Fig. 4. IgE-binding capacities of the natural and recombinant allergens Bet v 1 and Phl p 1 determined by EAST inhibition. (A) Inhibition curves obtained with nPhl p 1 and rPhl p 1 variants as inhibitors for nPhl p 1 specific IgE. (B) Inhibition curves obtained with nBet v 1 and rBet v 1 variants as inhibitors for nBet v 1 specific IgE.

### 3.3. SEC of crude extracts and natural purified allergens

Crude extract of birch pollen (*B. verrucosa*), natural purified Bet v 1 and the recombinant variants, and crude extract of timothy grass pollen (*P. pratense*), nPhl p 1 and the recombinant Phl p 1 molecules were applied to SEC. Matrices were chosen to meet the requirements for the separation of proteins with molecular weights ranging from 10 to 70 kDa. As shown in Figs. 5 and 6A, chromatograms are characteristic for the extracts. In order to determine elution volumes of the allergens Bet v 1 and Phl p 1, specific monoclonal antibodies (mabs) were used for tracing of these molecules in fractions obtained by separation of the crude extracts (bar overlays in Figs. 5 and 6A). Elution volumes of fractions reacting with the antibodies were equivalent to those obtained with the purified molecules, indicating that the purification of the natural proteins did not affect their migration and in consequence, conformational changes were not detectable (Figs. 5 and 6B, Table 1).

#### 3.3.1. SEC of natural Phl p 1 and recombinant variants

The peak of natural Phl p 1 eluted with 55.15 ml, which is very close to the elution volume of 55.20 ml obtained for Phl p 1 fractions of the extract (Fig. 5A,B, Table 1). While the nPhl p 1 peak appeared with slight tailing and small amounts of impurities with higher molecular weights were detectable, the shape of the rPhl p 1 CF peak was symmetric and did not show any impurities. Moreover, rPhl p 1 eluted with 55.22 ml, indicating high conformity with its natural counterpart (Fig. 5C). Compared with rPhl p 1 CF, the other variant rPhl p 1 MF clearly differed in terms of its elution volume of 51.41 ml as well as in its shape, showing a small shoulder in the leading front of the peak (Fig. 5D). The distinct differences of elution volumes demonstrate a high efficiency of the separation, bearing in mind that the differences of apparent molecular weights were as small as 3 kDa as judged by SDS-PAGE analysis (Fig. 5D and Table 1). As shown in Table 1, elution volumes differed in the same way when a Superdex 75 column was used. In

Table 1  
Elution volumes of natural and recombinant Bet v 1 and Phl p 1

| Protein             | Elution volume (ml)           |                           |                           |
|---------------------|-------------------------------|---------------------------|---------------------------|
|                     | Column 1<br>(Sephacryl S-100) | Column 2<br>(TSK 2000 SW) | Column 3<br>(Superdex 75) |
| nBet v 1 (extract)  | 64.64 ± 0.2                   | 17.20 ± 0.1               | n.d.                      |
| nBet v 1 (purified) | 64.54 ± 0.2                   | 17.17 ± 0.1               | n.d.                      |
| rBet v 1 CF         | 64.74 ± 0.2                   | 17.19 ± 0.1               | n.d.                      |
| rBet v 1 MF         | 52.22 ± 0.2                   | 16.02 ± 0.1               | n.d.                      |
| nPhl p 1 (extract)  | 55.20 ± 0.2                   | 16.25 ± 0.1               | 56.40 ± 0.2               |
| nPhl p 1 (purified) | 55.15 ± 0.2                   | 16.26 ± 0.1               | 56.50 ± 0.2               |
| rPhl p 1 CF         | 55.22 ± 0.2                   | 16.30 ± 0.1               | 56.48 ± 0.2               |
| rPhl p 1 MF         | 51.41 ± 0.2                   | 16.21 ± 0.1               | 52.80 ± 0.2               |

Extracts and purified proteins were applied to SEC. Elution volumes of Bet v 1 and Phl p 1 in the extracts were determined by using specific mabs for detection (refer to Figs. 5 and 6). Each preparation was run three-fold using three different matrices.

n.d.: not determined.

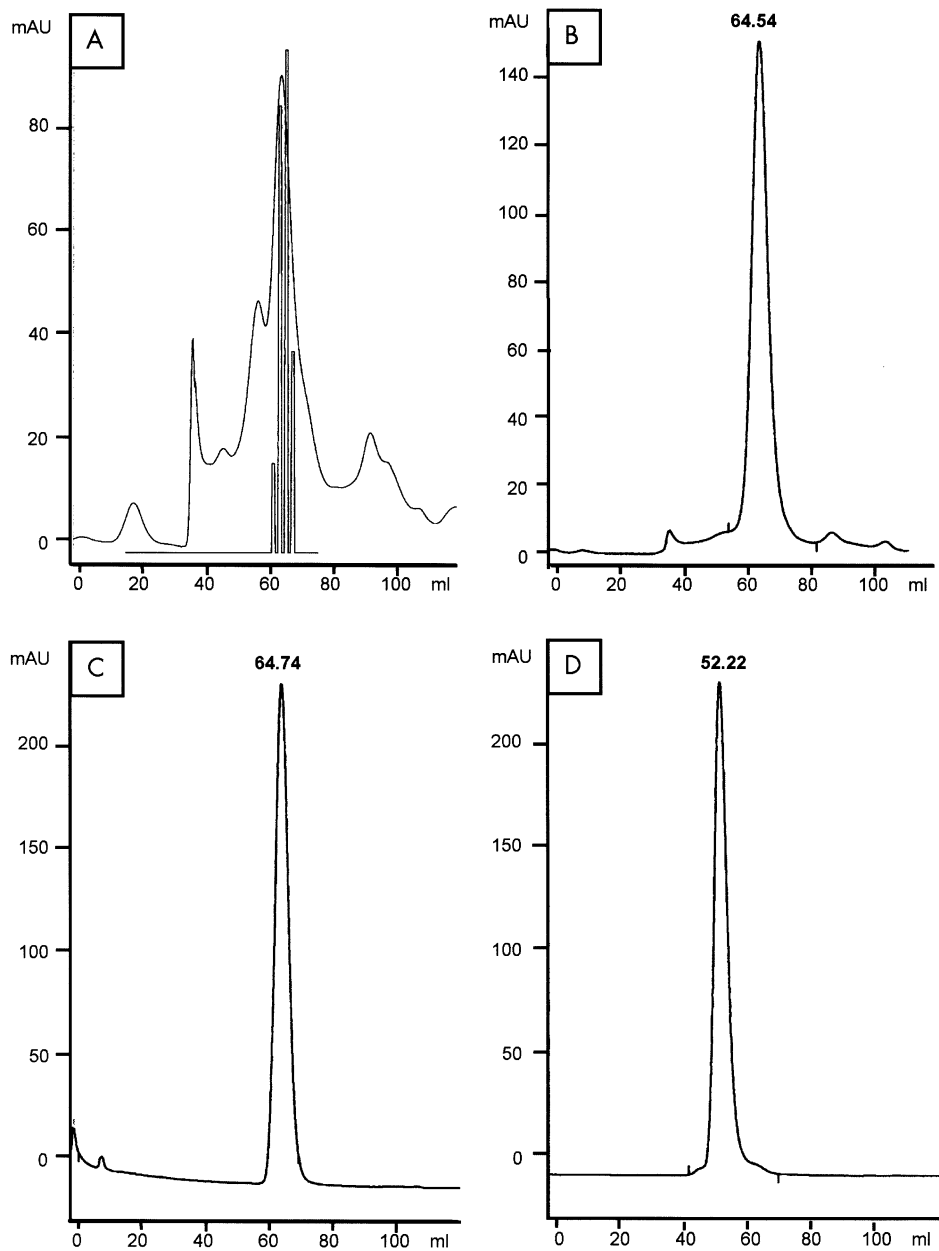


Fig. 5. Separation of birch pollen extract and major allergen Bet v 1 by size exclusion chromatography using Sephacryl S-100 in a  $16 \times 600$  mm column. (A) Chromatogram of birch pollen extract (line). Fractions were collected (each 0.5 ml) and analyzed with respect to Bet v 1 content (bars). (B) Chromatogram of affinity purified natural Bet v 1. (C) Chromatogram of rBet v 1 CF. (D) Chromatogram of rBet v 1 MF.

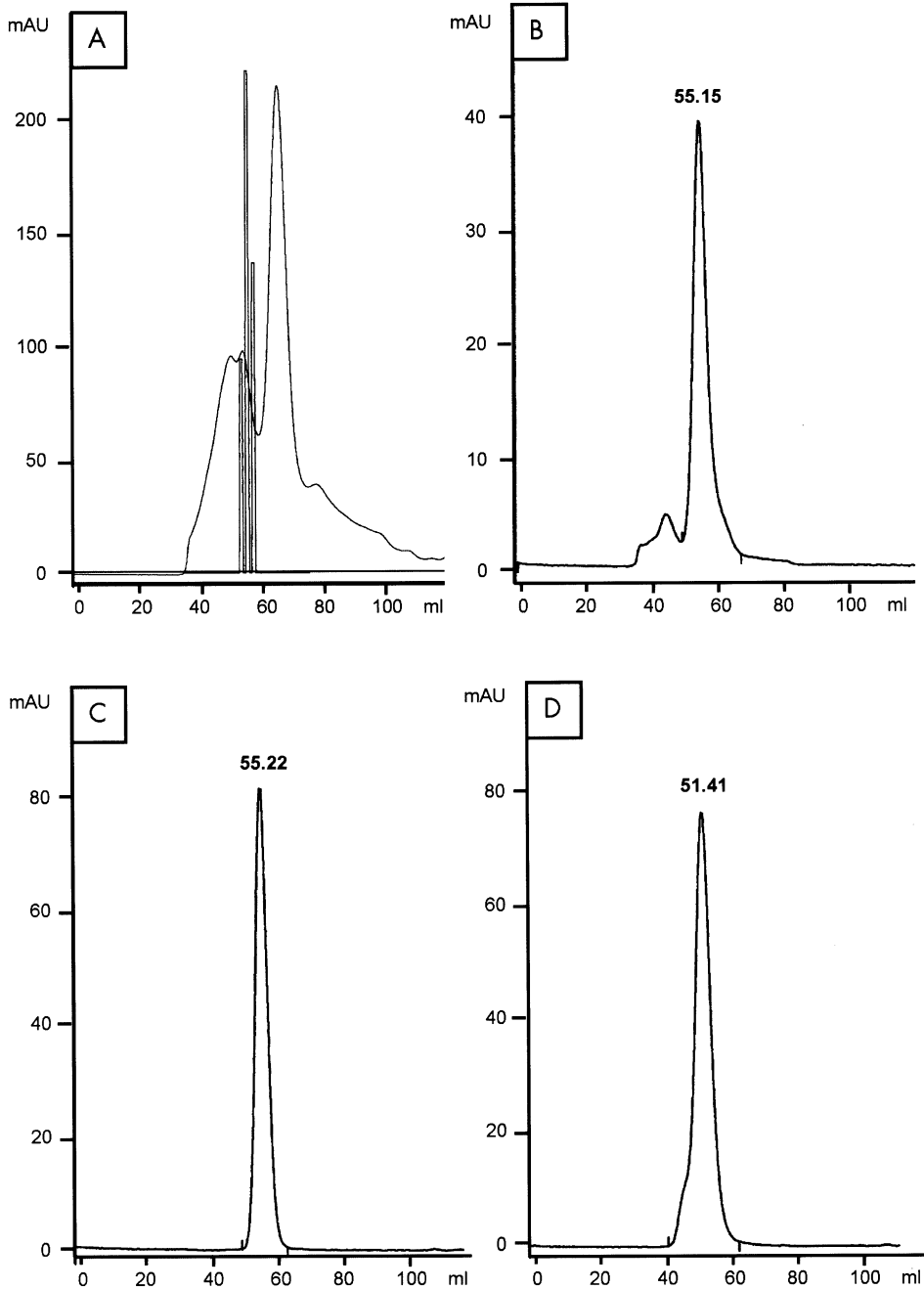


Fig. 6. Separation of timothy grass pollen extract (*P. pratense*) and major allergen Phl p 1 by size exclusion chromatography using Sephacryl S-100 in a  $16 \times 600$  mm column. (A) Chromatogram of grass pollen extract (line). Fractions were collected (each 0.5 ml) and analyzed with respect to Phl p 1 content (bars). (B) Chromatogram of purified natural Phl p 1. (C) Chromatogram of rPhl p 1 CF. (D) Chromatogram of rPhl p 1 MF.

contrast, by using a TSK 2000 SW column for these separations, elution volumes of the recombinant variants did not differ significantly. The unexpected uniformity of elution volumes might be due to the less pronounced alteration of the molecular size compared with the rBet v 1 variants (see below), which did not influence the interaction of the rPhl p 1 variants with the silica based matrix in the same way as with sephacryl or sephadex matrices.

### 3.3.2. SEC of natural Bet v 1 and recombinant variants

Elution volumes of nBet v 1 either in the crude extract or as a purified protein gave almost equivalent values, with 64.64 ml and 64.54 ml, respectively, confirming the efficiency of the affinity purification (Table 1 and Fig. 6A,B). In contrast, differences between the recombinant variants appeared very clearly, resulting in 64.74 ml elution volume for rBet v 1 CF and 52.22 ml for rBet v 1 MF (Table 1 and Fig. 6C,D). Differences between these variants were only detectable by immunological methods, as molecular weights of the molecules were not distinguishable by SDS-PAGE analysis. This might be due to the denaturation prior to separation by SDS, indicating conformational differences rather than differences of molecular weight. As shown in Table 1, these differences were also found by using a TSK 2000 SW column.

In conclusion, recombinant variants of Phl p 1 and Bet v 1 were found to be different in their IgE-binding capacities, considered to be the most important property of allergens. Comparable differences had been shown with folding variants of the recombinant wasp venom allergen Ves v 5 [9].

While the common analysis by SDS-PAGE and immunoblot, which should be able to detect such crucial differences, does not prove to be effective, SEC gave reliable results. Moreover, not only distinct differences of elution volumes but also the determination of protein amounts or purity is provided. Whether the finding that distinct differences of elution volumes of recombinant and natural proteins equate with different IgE-binding capacities in general has to be established by further investigations. Nevertheless, for the quality control of rBet v 1 and rPhl p 1, SEC is considered to be the most reliable method, most likely superior to SDS-PAGE analysis, even when used in combination with immunoblots.

## 4. Simplified description of method and application

The important characterization and quality control of recombinant allergens can be powerful conducted by size exclusion chromatography. In a first step, either the natural allergen extract with subsequent detection of the concerning allergen or purified natural allergen should be separated in order to provide a reference. The second step is the separation of the corresponding recombinant allergen followed by comparison of the elution volumes or retention times. Deviations from the elution volume of the natural allergen indicate different conformation of the recombinant molecule, having in turn strong influences on the IgE-binding capability, which is especially important for allergens.

In addition, the method allows quantification by using the peak area and detection of impurities such as host cell proteins and dimers or multimers [22]. The solubility of the

preparations, also in buffer systems different from those used for production and storage, would also be verified. Taking together, once a characterization of the recombinant allergen with a panel of test methods is carried through, size exclusion chromatography should routinely be performed to substantiate at least three highly important assays: IgE-binding property, purity and quantity. It is therefore suggested to perform the size exclusion chromatography as a primary must for every recombinant allergen preparation.

## Acknowledgements

The excellent technical assistance of Sandra Sulewski and Claudia Fritz is gratefully acknowledged.

## References

- [1] Johansson SGO, Hourihane JO'B, Bousquet J, et al. A revised nomenclature for allergy. *Allergy* 2001;56:813–24.
- [2] Wahl R, Meineke D, Maasch HJ. Molecular weight determination of allergen extracts and isolation of allergenic molecules by high-performance liquid chromatography. *J Chromatogr* 1987;397:307–12.
- [3] Scheiner O, Kraft D. Basic and practical aspects of recombinant allergens. *Allergy* 1995;50:384–91.
- [4] Valenta R, Vrtala S, Laffer S, Spitzauer S, Kraft D. Recombinant allergens. *Allergy* 1998;53:552–61.
- [5] Schmid-Grendelmeier P, Cramer R. Recombinant allergens for skin testing. *Int Arch Allergy Immunol* 2001;125:96–111.
- [6] Niederberger V, Stubner P, Spitzauer S, et al. Skin test results but not serology reflect immediate type respiratory sensitivity: a study performed with recombinant allergens molecules. *J Invest Dermatol* 2001; 117:848–51.
- [7] Nikolaizik WH, Weichel M, Blaser K, Cramer R. Intracutaneous test with recombinant allergens in cystic fibrosis patients with allergic bronchopulmonary aspergillosis and *Aspergillus* allergy. *Am J Respir Crit Care Med* 2002;165:916–21.
- [8] Schramm G, Kahlert H, Suck R, et al. Allergen engineering: variants of the timothy grass pollen allergen Phl p 5b with reduced IgE-binding but conserved T cell reactivity. *J Immunol* 1999;162:2406–14.
- [9] Suck R, Weber B, Kahlert H, Hagen S, Cromwell O, Fiebig H. Purification and immunobiochemical characterization of folding variants of the recombinant major wasp allergen Ves v 5 (Antigen 5). *Int Arch Allergy Immunol* 2000;121:284–91.
- [10] Suck R, Hagen S, Cromwell O, Fiebig H. Rapid and efficient purification of *Phleum pratense* major allergens Phl p 1 and group Phl p 2/3 using a two step procedure. *J Immunol Methods* 1999;229: 73–80.
- [11] Harlow E, Lane D. Preparing protein A bead-antibody affinity columns—direct coupling in high salt. *Antibodies—a laboratory manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory; 1988. p. 524–5.
- [12] Kahlert H, Weber B, Teppke M, Wahl R, Cromwell O, Fiebig H. Characterization of major allergens of *Parietaria officinalis*. *Int Arch Allergy Immunol* 1996;109:141–9.
- [13] Petersen A, Schramm G, Bufer A, Schlaak M, Becker WM. Structural investigations of the major allergen Phl p 1 on the complementary DNA and protein level. *J Allergy Clin Immunol* 1995;95:987–94.
- [14] Breiteneder H, Pettenburger K, Bito A, et al. The gene coding for the major birch pollen allergen Bet v 1, is highly homologous to a pea disease resistance response gene. *EMBO J* 1989;8:1935–8.
- [15] Suck R, Hagen S, Slamal H, et al. Recombinant hypoallergenic Bet v 1 derivatives suitable for clinical application [Abstract]. *Allergy* 2001;56:261.
- [16] Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5.

- [17] Grobe K, Becker W-M, Schlaak M, Petersen A. Grass group I allergens ( $\beta$ -expansins) are novel, papain-related proteinases. *Eur J Biochem* 1999;263:33–40.
- [18] Laffer S, Valenta R, Vrtala S, et al. Complementary DNA cloning of the major allergen *Phl p 1* from timothy grass (*Phleum pratense*); recombinant *Phl p 1* inhibits IgE binding to group I allergens from eight different grass species. *J Allergy Clin Immunol* 1994;94:689–98.
- [19] Petersen A, Mundt C. Investigations on the carbohydrate moieties of glycoprotein allergens. *J Chromatogr B Biomed Sci Appl* 2001;756:141–50.
- [20] Anliker MD, Reindl J, Vieths S, Wuthrich B. Allergy caused by ingestion of persimmon (*Diospyros kaki*): detection of specific IgE and cross-reactivity to profilin and carbohydrate determinants. *J Allergy Clin Immunol* 2001;107:718–23.
- [21] Slater JE, Pastor RW. The determination of equivalent doses of standardized allergen vaccines. *J Allergy Clin Immunol* 2000;105:468–74.
- [22] Meyer RK, Lustig A, Oesch B, et al. A monomer–dimer equilibrium of a cellular prion protein (PrPC) not observed with recombinant PrP. *J Biol Chem* 2000;275:38081–7.