Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography B, 787 (2003) 357–368
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Purification strategy for recombinant Phl p 6 is applicable to the natural allergen and yields biochemically and immunologically comparable preparations

Roland Suck*, Bernhard Weber, Brigitte Schäffer, Ellen Diedrich, Timo Kamionka, Helmut Fiebig, Oliver Cromwell

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

Received 3 September 2002; received in revised form 22 November 2002; accepted 26 November 2002

Abstract

The recombinant major grass pollen allergen Phl p 6 has been expressed with a N-terminal 6×His-tag sequence and subsequently purified using nickel-chelating Sepharose. After cleavage of the tag-sequence, a second pass over the affinity chromatography revealed that even untagged rPhl p 6 bound tightly. In order to determine if that property is typical for Phl p 6, the natural allergen was purified in the same way starting with a grass pollen extract. Indeed, nPhl p 6 could be highly enriched in one step using nickel-chelating Sepharose. In addition to this new powerful purification method, the results provide further information in that the recombinant and natural allergens share a lot of properties, since biochemical characteristics are reflected in the purification strategies. The preparations of natural and recombinant Phl p 6 were used for comparative electrophoretic, chromatographic and immunological analysis which demonstrated high similarity.

Keywords: Recombinant allergen; Phl p 6

1. Introduction

Type I allergies [1] represent a major health problem in industrialized countries. Diagnosis of allergy and specific immunotherapy are currently carried out with allergen extracts containing the relevant allergenic proteins together with many nonallergenic components and carbohydrates. It is now possible to improve such preparations with regard to final composition, standardization, potency and stability with the increasing number of available recombinant allergens [2–4]. Since pollen from Poaceae grasses are the major cause of seasonal atopic allergy, the most important allergens [5] have been cloned and successfully expressed, including group 1 [6,7], group 5 [7,8], group 2 [9] and group 13 allergens [10].

In contrast to other major grass pollen allergens, group 6 allergens have been detected in only a few grass species [11,12], such as *Phleum pratense*. The 13-kDa allergen Phl p 6 was identified at the cDNA level [11] and exhibits sequence homology to group 5 allergens [11,12]. Together with group 1, 2 and 5 allergens, Phl p 6 is considered to be of particular

*Corresponding author. Tel.: +49-40-7276-5106; fax: +49-40-7276-5252.
E-mail address: roland.suck@allergopharma.de (R. Suck).

1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(02)00966-2

interest as a grass pollen-specific marker due to the absence of cross-reactive proteins in other plants [13].

However, recombinant allergens with potential application in diagnosis and therapy of allergic diseases require a careful and intensive comparison with the natural counterpart to provide high efficiency in diagnosis or a solid basis for the development of hypoallergenic variants [14]. Consequently, purified natural allergen is required, in addition to the recombinant allergen, especially when the recombinant allergen represents only one of several isoforms as is the case with Phl p 6 [15]. The purification methods for natural allergens described thus far are affinity chromatography using mAbs [16], reversed-phase HPLC [17,18] and a combination of different chromatographic steps [19].

In this study, recombinant Phl p 6 was expressed in bacteria as a fusion protein and subsequently purified via chelating Sepharose. After cleaving the His-tag, the processed rPhl p 6 surprisingly showed a significant affinity for the matrix. Basically the same procedure was subsequently used to purify natural Phl p 6 from a grass pollen extract. Using identical purification schemes for both the natural and recombinant allergens thus provides both material for comparative immunological investigations and a powerful biochemical characterization, since specific and individual properties of the molecules are the basis for the purification. In addition, it can be concluded that the structure and histidine content of the recombinant Phl p 6 represent those of the natural counterpart.

2. Experimental

2.1. Expression of rPhl p 6

The cDNA of Phl p 6 [11] was subcloned into a pProEx vector (Gibco–BRL, La Jolla, CA, USA) using the *Ehe* I/*Hin*d III restriction sites. After sequence confirmation, the construct was transformed in *E. coli* Bl 21 (Novagen, Bad Soden, Germany) cells. Expression was conducted by induction of a LB-liquid culture with isopropyl β-thiogalactoside (1 mmol/l) when cells were grown to an optical density of 0.6–1.0 measured at 600 nm.

2.2. Purification of rPhl p 6

After cell harvesting by centrifugation, cells were resuspended in 50 m*M* Na-phosphate buffer, 500 m*M* NaCl, pH 7.5 (CS-buffer) and lysed by ultrasonication followed by addition of benzonase (Merck, Darmstadt, Germany) to reduce viscosity. The cleared supernatant was directly applied to a 5 ml HiTrap chelating Sepharose column (Amersham Biosciences, Freiburg, Germany) with an average particle size of 34 μm, a capacity of 23 μmol Cu^{2+}/ml gel, previously loaded with nickel sulfate according to the manufacturer's instructions and equilibrated with CS-buffer at a flow-rate of 5 ml/min. Elution of bound proteins was conducted by a two-step elution using 100 m*M* imidazole in CB-buffer to remove impurities and 400 m*M* imidazole in CB-buffer to elute His-tagged rPhl p 6. In order to prepare the fusion protein for cleavage, a gelfiltration with Sephadex G-25 (Amersham) was carried out using a 2.6×60 cm XK-column (Amersham) with a flow-rate of 8 ml/min and 50 m*M* Tris–HCl, 0.5 m*M* EDTA, pH 8.0 as running buffer. The cleavage of the fusion protein was achieved by addition of 10 U TEV-Protease (Invitrogen, Karlsruhe, Germany) per mg eluted fusion protein followed by incubation for 20 h at 20 °C. Again, a G-25 gelfiltration with CB-buffer was carried out to provide the starting conditions for the second passage over chelating Sepharose in order to separate tagged and untagged rPhl p 6. The column was eluted by a linear gradient ranging from 0 to 500 m*M* imidazole in CB-Buffer over 10 column volumes. Final purification of the processed rPhl p 6 was achieved by gelfiltration with Superdex 75 (Amersham) packed in a 5.0×60 cm XK column with a flow-rate of 5 ml/min and 0.9% NaCl as running buffer.

2.3. Timothy pollen extracts

Five grams of timothy pollen (Allergon, Ängelholm, Sweden), previously defatted with petroleum ether followed by air drying, were suspended in 20 ml of 0.02 *M* Tris–HCl, 1 m*M* EDTA, pH 8.0 and incubated under agitation at room temperature for 30 min [20]. After centrifugation for 5 min at 22 000 *g* the supernatant was directly applied to a Sephadex G-25 gelfiltration instrument (column:

2.6×60 cm, approx. volume 300 ml, flow-rate 8 ml/min) in order to remove low molecular mass substances <5 kDa. Proteins were eluted using CB-buffer.

2.4. Purification of nPhl p 6

The low molecular mass depleted extract was applied onto a 5 ml HiPrep chelating Sepharose column. Bound proteins were eluted by a linear gradient from 0 to 500 m*M* imidazole in CB-Buffer over 10 column volumes, exactly as for the second passage of the recombinant allergen. Protein containing fractions were collected, adjusted to 1 *M* ammonium sulfate and applied onto an XK column (1.6×20 cm) filled with Phenyl Sepharose HP (Amersham Biosciences) equilibrated with 20 m*M* Tris–HCl, 1 *M* ammonium sulfate, pH 8.0. Elution was carried out with 20 m*M* Tris–HCl pH 8.0 by a linear gradient over 10 column volumes. Phl p 6 containing fractions were concentrated using a Centriprep-3 device (cut-off 3 kDa; Millipore, Eschborn, Germany) and finally purified by Superdex 75 gelfiltration and 0.9% NaCl as mobile phase.

2.5. Protein determination

Protein concentrations of recombinant and natural allergens were determined by optical density measurement at 280 nm using an extinction coefficient calculated according to Gill and von Hipple [21]. In the case of rPhl p 6, the coefficient was 0.43, which was also assumed for natural Phl p 6.

For control purposes, protein concentrations were also 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. The results in both cases were highly comparable and displayed minor deviations below 15%.

2.6. N-terminal sequencing

Microsequencing of purified proteins was carried out using a 473 A protein sequencer with on-line PTH amino acid analyzer (Applied Biosystems, Weiterstadt, Germany).

2.7. Analytical electrophoresis

Proteins were separated according their molecular size by SDS–PAGE (15% T, 4% C; size 120×80×0.8 mm^3) with the buffer system of Laemmli [22]. After electrophoresis, proteins were either stained or subsequently transferred onto a supported nitrocellulose membrane (Sartorius, Göttingen, Germany) by semi-dry blotting for 30 min at 2 mA/cm^2.

Separation of proteins according to charge, size and conformation was conducted by native PAGE, which was carried out exactly according to SDS–PAGE except that SDS and DTT was excluded.

Native analysis of proteins according to isoelectric points was carried out using agarose-based gels containing 4% (v/v) Servalyt (Boehringer Ingelheim Bioproducts, Heidelberg, Germany) with a pH-gradient ranging from 4.0 to 6.0.

2.8. Analytical RPC

Reversed-phase chromatography was carried out on a HPLC (Kontron Instruments, Neufahrn, Germany) equipped with a 540 Diode array detector and a Bio-Tek 525 pump system using a ET 250-4 Nucleosil 100-5 C_{18} PPN column (Macherey-Nagel, Dueren, Germany) with a particle size of 5 μm and dimensions of 4×250 mm. The column was equilibrated with distilled water/0.1% TFA at 1 ml/min, loaded with 50 μg protein and eluted using a three-step gradient (10–40% over 20 ml, 40% plateau over 5 ml, 40–60% over 20 ml) with acetonitrile/0.7% TFA (Merck). Detection was carried out at 220 nm.

2.9. EAST inhibition

For comparison of the relative IgE-reactivities of nPhl p 6 and rPhl p 6, an EAST inhibition assay was carried out as described [23]. Microtiter plates were coated with purified nPhl p 6 or rPhl p 6 (0.1 μg/well). Before loading to the microtiter plate, individual sera or serum pools obtained from grass pollen allergic subjects each diluted 1:2 were preincubated with serial dilutions of nPhl p 6 and rPhl p 6 (1.0×10^{-4} to 10 μg/ml). The allergenic activity of nPhl p 6 or rPhl p 6 was expressed as the amount of inhibitor (μg) necessary to obtain 50% inhibition.

Results are presented as means of duplicate determinations.

3. Results

3.1. Purification of rPhl p 6

The clarified *E. coli* cell homogenate containing soluble His-tagged Phl p 6 was applied to nickel chelating Sepharose and eluted with imidazol. After the first purification step His-Phl p 6 was estimated to be >90% pure. Subsequent addition of TEV-Protease resulted in approx. 50% cleavage. In order to separate the remaining fusion protein, the sample was subjected to nickel chelating Sepharose a second time (Fig. 1). It was expected to find cleaved rPhl p 6 in the flow-through fraction, as was the case for rPhl p 5a and rPhl p 5b (not shown). Surprisingly, the flow-through fraction contained no protein (Fig. 1A), whilst two peaks instead of one were eluted using a linear gradient. Electrophoretic separation identified peak 1 as the processed rPhl p 6 and peak 2 as the uncleaved fusion protein (Fig. 1B). It appeared that rPhl p 6 showed affinity for immobilized nickel, even without an artificial 6×His-tag. Regarding the sequence, rPhl p 6 contains three (EMBL accession no. O65868) or four (EMBL accession no. P43215) histidine residues that are presumably responsible for the observed affinity (Fig. 2).

Finally, a size exclusion on Superdex 75 served for polishing and buffer exchange (not shown). The yield of purified rPhl p 6 was more than 25 mg/l expression culture (shaking flask), though approx. 50% were lost due to incomplete cleavage of the fusion part.

3.2. Purification of nPhl p 6

In order to establish whether the observed affinity of rPhl p 6 to nickel chelating Sepharose is also attributable to the natural counterpart, a timothy grass pollen extract was subjected to the column (Fig. 3). The flow-through and peak fractions (Fig. 3A) were collected and subsequently analysed by SDS–PAGE (Fig. 3B) and subjected to immunological investigation using monoclonal antibodies (Fig. 3C). The flow-through fraction corresponded to the extract except that a 13-kDa and a 17-kDa protein were clearly depleted as judged by the protein patterns (arrows in Fig. 3B). These components eluted sharply with approx. 100 m*M* imidazole corresponding to 20% B. In addition, larger proteins of 55–60 kDa were also present in the peak fraction. Immunological analysis revealed that both major allergens Phl p 1 (33 kDa) and Phl p 5 (30–35 kDa) were not adsorbed to the column. The same was observed for Phl p 4 using mabs 5H1 and 3C4 [24]. In contrast, Phl p 6 could not be detected in the flow-through but in the peak fraction, indicating complete binding. The monoclonal antibody directed against Phl p 13 (55–60 kDa) identified the major allergen in both fractions.

After addition of ammonium sulfate to 1 *M* final concentration, the peak fraction was applied to HIC on phenyl Sepharose in order to separate Phl p 6 from remaining impurities, hence the 17-kDa protein and Phl p 13 (Fig. 4A). Under these conditions, Phl p 13 is expected in the flow through fraction [20]. Phl p 6 and the 17-kDa protein could be efficiently segregated using a linear gradient over 10 CV. Final purification and desalting of pooled fractions was achieved by gel filtration on Superdex 75 (Fig. 4B). The final yield of highly purified nPhl p 6 was 1 mg/g extracted pollen. Identification and purity of Phl p 6 was confirmed by N-terminal sequencing.

3.3. Comparative analysis of nPhl p 6 and rPhl p 6

Electrophoretic analysis was conducted by three different methods, SDS–PAGE, isoelectric focusing and native PAGE. The SDS–PAGE separation of nPhl p 6 (Fig. 5A, Lane 1) and rPhl p 6 (Fig. 5A, Lane 2) demonstrated the purity of both proteins, though the natural allergen migrated as a slightly diffuse band. Two Western blots obtained from similar gels were investigated with the Phl p 6 specific mab Bo12 (kindly provided by Dr A. Petersen, Borstel, Germany) and a serum pool obtained from grass pollen allergic patients ($n=3$), respectively. In each case a clear detection was observed, identifying both proteins as the major allergen Phl p 6. In order to investigate possible isoforms of the natural allergen, an isoelectric focusing followed by immunological investigation using

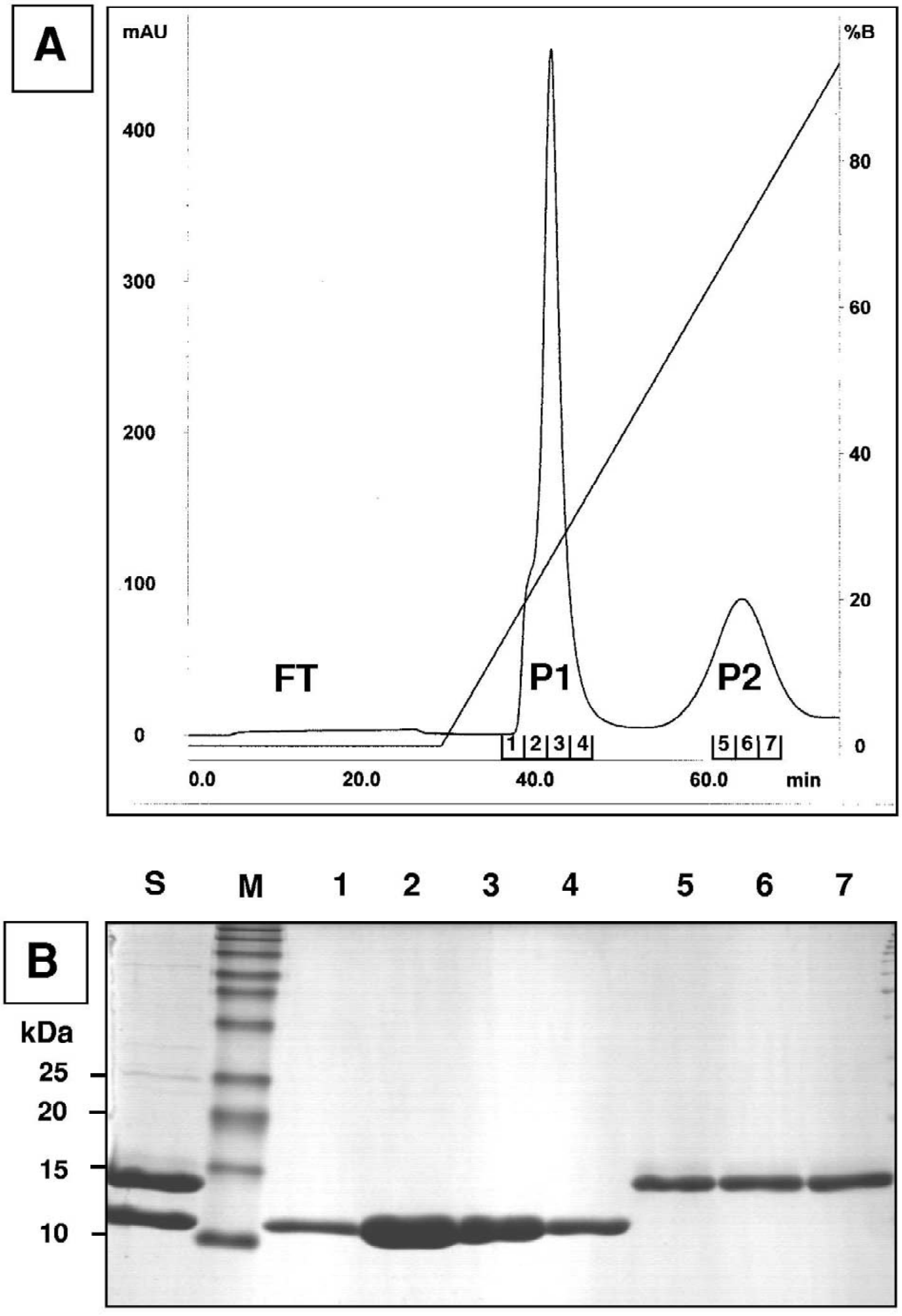


Fig. 1. Purification of rPhl p 6. (A) Chromatogram of purified and partially cleaved 6×His-tagged rPhl p 6 applied to nickel chelating Sepharose. Elution was carried out with a linear 0–500 m*M* imidazole gradient. Fractions used for subsequent SDS–PAGE analysis are indicated. (B) SDS–PAGE analysis of the applied sample (S) and collected fractions from peak 1 (P1) and peak (P2). Size estimation was conducted by comparison with a prestained molecular mass marker (M).

```
MSYYHHHHHHDYDIPTTENLYFQGGKATTEEQKLIEDIN
ASFRAAMATTANVPPADKYKTFEAAFTVSSKRNLADAVS
KAPQLVPKLDEVYNAAYNAADHAAPEDKYEAFVLHFSEA
LHIIAGTPEVHAVKPGA
```

Fig. 2. Amino acid sequence of 6×His-tagged rPhl p 6. Histidine residues are highlighted by underlining and hatching. The arrow indicates the cleavage site of TEV-protease.

Bo12 was carried out (Fig. 5B). The natural Phl p 6 is comprised of three major isoforms with isoelectric points ranging from 6.1 to 5.2, and rPhl p 6 obviously represents one form with a p*I* of 6.1. Using native PAGE, proteins were separated according to size, charge and shape, providing even more detailed information (Fig. 5C). Again, three major bands were detected in the case of nPhl p 6 by Coomassie staining and specific detection by mab Bo 12. The recombinant Phl p 6 corresponded to the slow migrating form.

Reversed-phase analysis of the recombinant (Fig. 6A) and the natural Phl p 6 (Fig. 6B) resulted in single peaks with identical retention times of 27.5 min, enabling to conclude high purity and similarity.

Finally, allergenic activity was compared using an EAST-inhibition assay. The microtiter plate was coated with nPhl p 6 or rPhl p 6 and incubated with three individual sera already used as a pool in Fig. 5A and two serum pools each preadsorbed with increasing amounts of either nPhl p 6 or rPhl p 6 (Fig. 7, Table 1). Though the amount of nPhl p 6 inhibitor needed to obtain 50% inhibition was in each case approx. twice as much compared with rPhl p 6, the parallel curves clearly demonstrated a high comparability in IgE-reactivity between the natural and recombinant major allergen Phl p 6. The systematic minor difference between n- and rPhl p 6 concerning the 50% inhibition value can be attributed to the overall variability of the assay and are judged to be not significant. However, epitope structures are identical as demonstrated by these data.

4. Discussion

Many of the problems associated with using natural allergenic products for allergy diagnosis and treatment can be overcome with the use of recombinant allergens. Before application, a thorough comparison between the recombinant and natural allergen is necessary to ensure most possible efficiency and safety. A simple method to investigate many properties is to apply the same or similar purification methods for both proteins. In the case of the important major grass pollen allergen Phl p 6 a significant affinity of the molecule to nickel chelating Sepharose was detected along with the purification of rPhl p 6. It may be supposed that with respect to relevant amino acids, e.g. histidine, composition and location are identical, since accessibility by the matrix is a requirement for binding. Conformation in turn is an important factor for IgE-binding, the fundamental definition for an allergen. The comparable shapes of n- and rPhl p 6 were also confirmed by EAST-inhibition analysis. Though different isoforms of nPhl p 6 have been described [15,19], all forms of the natural allergen could be enriched by chelating chromatography. Taken together, sufficient biochemical and immunological evidence are provided to conclude that nPhl p 6 may be substituted by rPhl p 6. Therefore, rPhl p 6 may be used for at least diagnostic purposes, as suggested by Kazemi-Shirazi et al. [13].

Regarding specific immunotherapy, T-cell reactivity and the ability to modulate T-cell responses [25] as well as blocking IgG antibodies [26] are considered to play significant roles. The availability of purified natural allergen as obtained by the method described above is essential to investigate reactivity of rPhl p 6 in both respects.

Chelating Sepharose has already been successfully used to purify Phl p 5 [27], composed of Phl p 5a and Phl p 5b [28]. Interestingly, though Phl p 5 and Phl p 6 show a high degree of sequence homology [11], Phl p 6 and other proteins were obtained in only minor amounts, therefore described as impurities. A reason for this might be due to the attached copper instead of nickel. However, another impurity detected by Matthiesen and Löwenstein [27] was an IgE-reactive 55- to 60-kDa protein, obviously the recently identified Phl p 13 [10,29], which is very similar to this report.

In general, copper provides stronger affinity to one or more histidine and tryptophan residues, whereas nickel tends to have an increased selectivity. It is interesting to note that neither Phl p 5a nor Phl p 5b

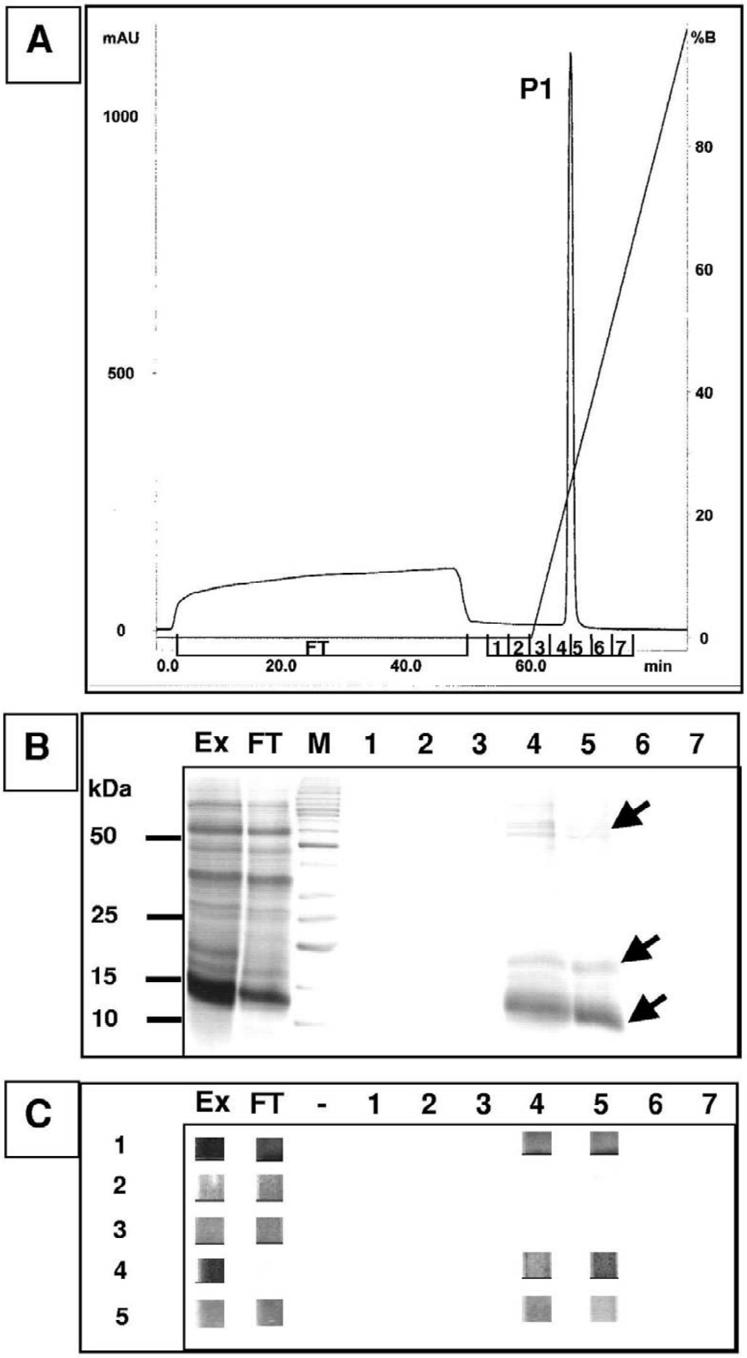
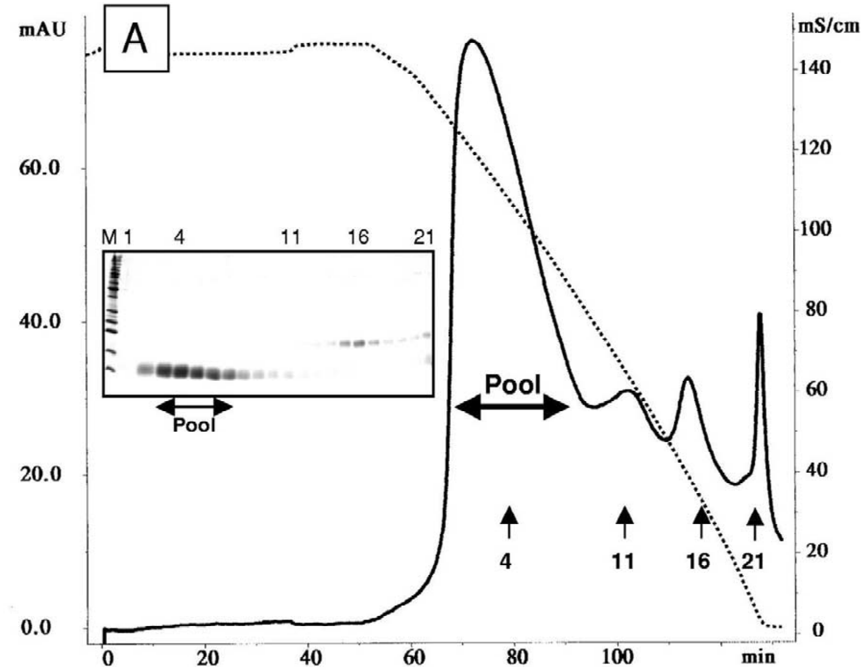


Fig. 3. Purification of nPhl p 6 by affinity chromatography. (A) Chromatographic profile of a timothy grass pollen extract applied onto nickel chelating Sepharose and subsequent elution with a linear 0–500 m*M* gradient. The flow-through (FT) and eluting fractions 1–7 of peak 1 (P1) were collected. (B) SDS–PAGE investigation of fractions indicated in (A). For comparison, the applied sample (Ex) was run on the same gel. Arrows indicate eluted proteins in peak 1 (P1). (C) Immunological investigation of immobilized extract (Ex), the flow-through (FT) and eluted proteins in fractions 1–7 with 1, protein staining by Auro dye; 2, mab 1D11 directed against Phl p 5 [24]; 3, mab 3G5 directed against Phl p 1; 4, mab Bo12 directed against Phl p 6; and 5, mab AF6 directed against Phl p 13 [29].



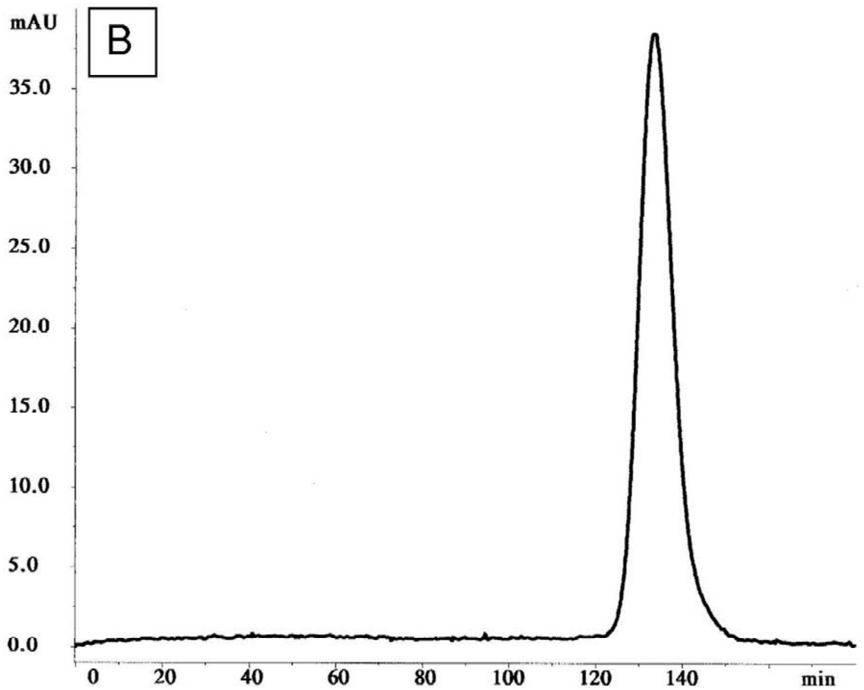


Fig. 4. Final purification of nPhl p 6 by HIC and SEC. (A) The peak fractions 4 and 5 obtained in Fig. 3A were applied onto Phenyl Sepharose and eluted with a linear gradient ranging from 1000 to 0 m*M* ammonium sulfate. Protein containing fractions were subjected to SDS–PAGE analysis as indicated by arrows (insert). (B) Final polishing of pooled nPhl p 6 containing fractions (fraction 3–7) was conducted by gelfiltration on Superdex 75.

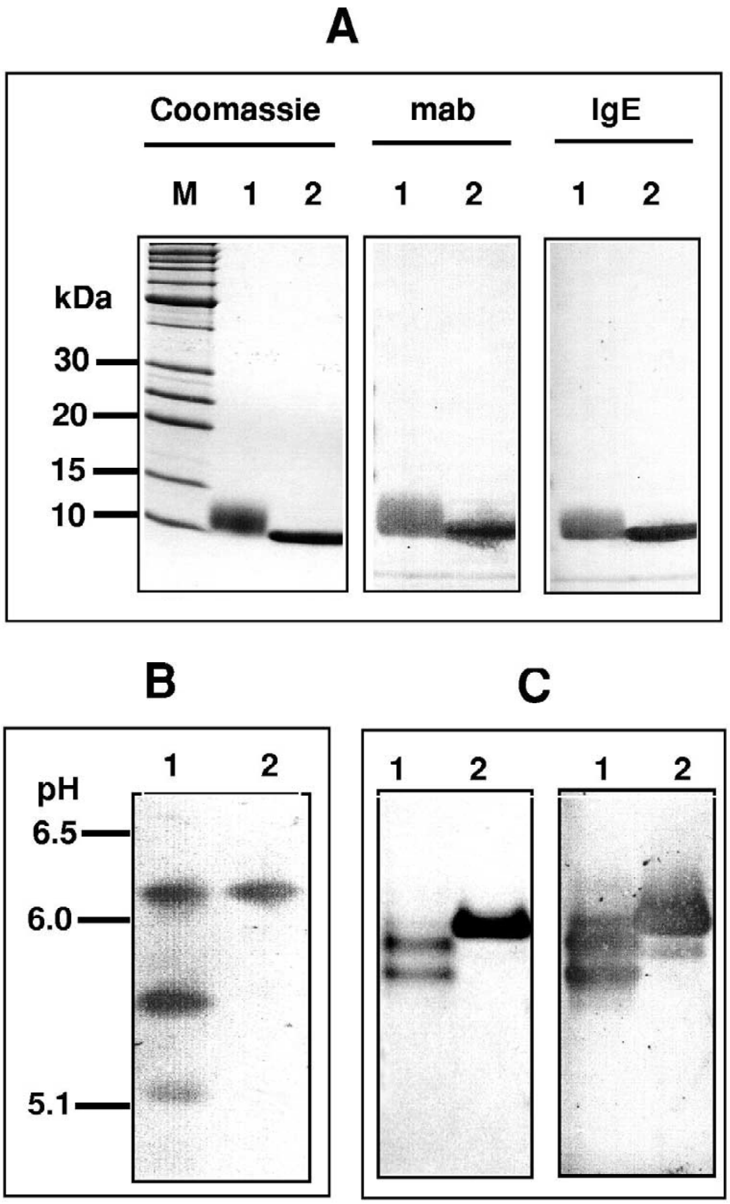


Fig. 5. Electrophoretic analysis of nPhl p 6 and rPhl p 6. (A) SDS–PAGE with separated molecular mass marker (M), nPhl p 6 (lane 1) and rPhl p 6 (lane 2) was either Coomassie-stained or, after blotting similar gels onto nitrocellulose, investigated by Phl p 6 specific mab Bo12 and serum IgE. (B) Isoelectric focusing of nPhl p 6 (lane 1) and rPhl p 6 (lane 2) followed by incubation with mab Bo12. (C) Separation of nPhl p 6 (lane 1) and rPhl p 6 (lane 2) by native PAGE followed by Coomassie staining (left side) and mab Bo12 incubation (right side).

contain Trp and only one histidine, which is obviously sufficient to provide affinity to copper. Phl p 6 contains three to four histidine residues and could not be enriched by copper chelating Sepharose, whereas even more selective conditions using nickel led to almost complete separation.

Taken together, the use of identical relevant purification steps for both the recombinant and

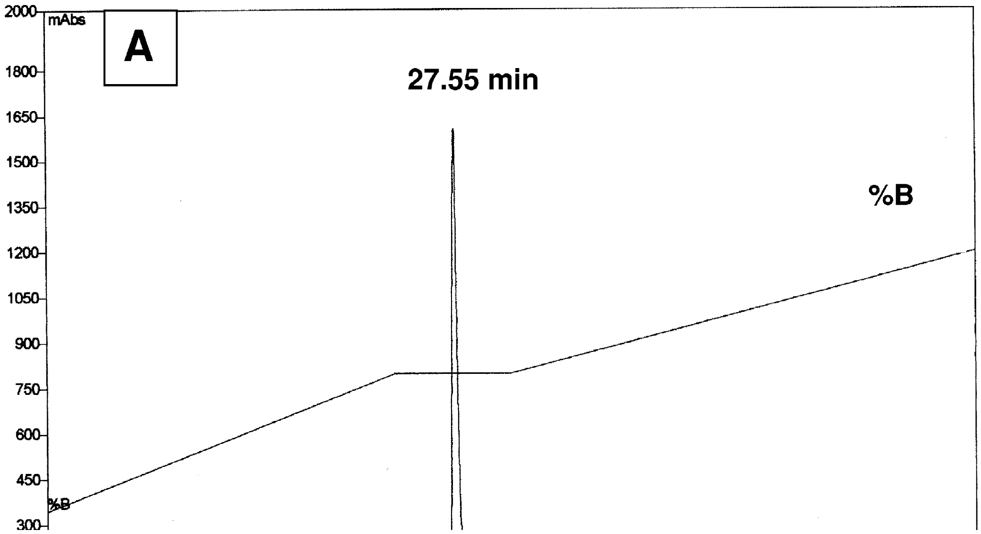


Fig. 6. RP-HPLC analysis of (A) rPhl p 6 and (B) nPhl p 6. In each case 50 μg were applied onto a Nucleosil 100-5 C_{18} PPN column and eluted using the indicated gradient (%B).

Table 1
Results of EAST inhibition assay using individual and pool sera derived from allergic patients

Serum	Solid phase: rPhl p 6		Solid phase: nPhl p 6	
	Inhibitor rPhl p 6	Inhibitor nPhl p 6	Inhibitor rPhl p 6	Inhibitor nPhl p 6
II 19	0.0032	0.0048	0.0045	0.0070
II 53	0.039	0.081	0.025	0.053
II 68	0.017	0.032	0.009	0.016
Pool 1 ($n=11$)	0.42	0.72	0.12	0.23
Pool 2 ($n=16$)	0.019	0.028	0.015	0.026

Values represent the amount of inhibitor (μg) necessary to obtain 50% inhibition in the respective assay.

Acknowledgements

The authors thank PD Dr Arnd Petersen for the determination of N-terminal sequences and for providing monoclonal antibodies. The excellent technical assistance of Marion Stock is gratefully acknowledged.

References

[1] S.G. Johansson, J.O'B. Hourihane, J. Bousquet, C. Bruijnzeel-Koomen, S. Dreborg, T. Haahtela, M.L. Kowalski, N. Mygind, J. Ring, P. van Cauwenberge, M. van Haage-Hamsten, B. Wuthrich, Allergy 56 (2001) 813.
[2] R. Valenta, S. Vrtala, S. Laffer, S. Spitzauer, D. Kraft, Allergy 53 (1998) 552.
[3] M.D. Chapman, A.M. Smith, L.D. Vailes, L.K. Arruda, V. Dhanaraj, A. Pomés, J. Allergy Clin. Immunol. 106 (2000) 409.
[4] P. Schmid-Grendelmeier, R. Crameri, Int. Arch. Allergy Immunol. 125 (2001) 96.
[5] C. Suphioglu, Clin. Exp. Allergy 30 (2000) 1335.
[6] A. Petersen, G. Schramm, A. Bufe, M. Schlaak, M.W. Becker, J. Allergy Clin. Immunol. 95 (1995) 987.
[7] S. Vrtala, M. Susani, W.R. Sperr, P. Valent, S. Laffer, C. Dolecek, D. Kraft, R. Valenta, J. Allergy Clin. Immunol. 97 (1996) 781.
[8] E. van Oort, P.G. de Heer, P. Lerouge, L. Faye, R.C. Aalberse, R. van Ree, Int. Arch. Allergy Immunol. 126 (2001) 196.
[9] C. Dolecek, S. Vrtala, S. Laffer, P. Steinberger, D. Kraft, O. Scheiner, R. Valenta, FEBS Lett. 335 (1993) 299.
[10] R. Suck, A. Petersen, S. Hagen, O. Cromwell, W.M. Becker, H. Fiebig, Clin. Exp. Allergy 30 (2000) 324.
[11] A. Petersen, A. Bufe, G. Schramm, M. Schlaak, W.M. Becker, Int. Arch. Allergy Immunol. 108 (1995) 55.
[12] S. Vrtala, S. Fischer, M. Grote, L. Vangelista, A. Pastore, R. Sperr, P. Valent, R. Reichelt, D. Kraft, R. Valenta, J. Immunol. 163 (1999) 5489.
[13] L. Shirazi-Kazemi, V. Niederberger, B. Linhart, J. Lidholm, D. Kraft, R. Valenta, Int. Arch. Allergy Immunol. 127 (2002) 259.
[14] M.B. Singh, N. de Weerd, P.L. Bhalla, Int. Arch. Allergy Immunol. 119 (1999) 75.
[15] A. Petersen, A. Bufe, M. Schlaak, M.W. Becker, Int. Arch. Allergy Immunol. 108 (1995) 49.
[16] Y. Boutin, P. Lamontagne, J. Boulanger, C. Brunet, J. Hébert, Int. Arch. Allergy Immunol. 112 (1997) 218.
[17] W.D. Müller, T. Karamifilov, A. Bufe, B. Fahlbusch, I. Wolf, L. Jäger, Int. Arch. Allergy Immunol. 109 (1996) 352.
[18] B. Fahlbusch, W.D. Müller, O. Rudeschko, L. Jäger, O. Cromwell, H. Fiebig, Clin. Exp. Allergy 28 (1998) 799.
[19] H. Löwenstein, Allergy 33 (1978) 30.
[20] R. Suck, S. Hagen, O. Cromwell, H. Fiebig, J. Immunol. Methods 229 (1999) 73.
[21] S.C. Gill, P.H. von Hippel, Anal. Biochem. 182 (1989) 319.
[22] U.K. Laemmli, Nature 227 (1970) 680.
[23] G. Schramm, H. Kahlert, R. Suck, B. Weber, H.T. Stüwe, W.D. Müller, A. Bufe, W.M. Becker, M.W. Schlaak, L. Jäger, O. Cromwell, H. Fiebig, J. Immunol. 162 (1999) 2406.
[24] B. Fahlbusch, W.D. Müller, C. Diener, L. Jäger, Clin. Exp. Allergy 23 (1993) 51.
[25] H. Kahlert, E. Grage-Griebenow, H.T. Stüwe, O. Cromwell, H. Fiebig, J. Immunol. 165 (2000) 1807.
[26] R. van Ree, R.C. Aalberse, Int. Arch. Allergy Immunol. 106 (1995) 250.
[27] F. Matthiesen, H. Löwenstein, Clin. Exp. Allergy 21 (1991) 297.
[28] K. Gehlhar, A. Petersen, G. Schramm, W.M. Becker, M. Schlaak, A. Bufe, Eur. J. Biochem. 247 (1997) 217.
[29] A. Petersen, R. Suck, S. Hagen, O. Cromwell, H. Fiebig, W.M. Becker, J. Allergy Clin. Immunol. 107 (2001) 856.
[30] R. Suck, A. Nandy, B. Weber, M. Stock, H. Fiebig, O. Cromwell, Allergy 57 (2002) 821.