Major allergen *Phl p* Vb in timothy grass is a novel pollen RNase

A. Bufe[a,*], G. Schramm[a], M.B. Keown[b], M. Schlaak[a], W.-M. Becker[a]

[a]*Research Institute Borstel, Allergology, Parkallee 22, D-23845 Borstel, Germany*
[b]*The Randall Institute, King's College London, 26–29 Drury Lane, London, WC2B SRL, UK*

Received 12 February 1995

Abstract A cDNA coding for the major group V allergen *Phl p* Vb was isolated from a timothy grass pollen cDNA library by immunoscreening with a specific monoclonal antibody. It was discovered for the first time that the recombinant *Phl p* Vb pollen allergen after expression and purification has ribonuclease activity. High homology of *Phl p* Vb to other group V allergens in grass pollen indicates similar function. By RNase activity gel of natural pollen extract of timothy grass and consecutive Western blot analysis of the excised proteins, the RNase active bands were shown to be group V allergens. Additionally it was demonstrated that an homologous protein to *Phl p* Vb in the mother plant could be induced by salicylic acid. This indicates that group Vb allergens may be involved in host–pathogen interactions because in pollen they are quickly exported RNases and in the mother plant they depend on a hormone which is related to expression of plant resistance genes.

Key words: Major allergen; Timothy grass pollen; cDNA; RNase activity; Salicylic acid

1. Introduction

Major grass pollen allergens induce allergic rhinoconjunctivitis or bronchial asthma in more than 90% of grass pollen allergic and nearly 40% of all Type-I allergic patients [1]. The physiological function of these export proteins has not been clarified until now. In ray grass pollen, group V allergens are located in the starch granules [2,3]. The potency of these allergens is related to the fact that they can be released into the environment from the pollen with the starch granules and thus are small enough to enter the human airway to induce an IgE-mediated response in asthmatic patients [4]. These allergens represent a highly variable group of proteins which can be distinguished in timothy grass pollen by molecular weight into group Va = 38 kDa (more basic proteins) and group Vb = 32 kDa (more acidic proteins) [5,6]. Cloning and sequencing of group Va cDNAs revealed a homology of more than 85% among these allergens [7–9] indicating that they are isoforms of a gene family in related grasses. The physiological function of group V allergens in pollen grain is still not understood. It is proposed that some pollen allergens may play a role in recognition of pollen grain by its specific plant stigma inducing an inhibition reaction on the stigma surface in case of incompatibility between heterologous plants [10,11].

In this paper we report the complete cDNA and deduced amino acid sequence of *Phl p* Vb and describe, for the first time, that this allergen shows ribonuclease activity. By inducing timothy grass inflorescence with salicylic acid and consecutive Western blotting we present evidence that *Phl p* Vb is possibly related to host–pathogen interactions in the mother plant.

2. Materials and methods

2.1. Monoclonal antibodies (Mabs), immunoscreening

The cDNA library was constructed in a λ-Zap phage vector as described earlier [9]. Immunoscreening of the cDNA library was performed with Mab Bo1 [12], which recognizes *Phl p* Va and b in crude pollen extract. Mabs BG6 and Bo9 [13] identify only *Phl p* Va and *Phl p* Vb, respectively. Both are markers for internal group V allergen differentiation in timothy grass pollen.

Positive clones were plaque purified by several rounds of immunoscreening. Plasmid pBluescript was excised in vivo from isolated phages by co-infection with helper phage R408 (Strategene, La Jolla, USA), plated, and plasmids were amplified from single colonies, followed by plasmid preparation (Plasmid purification kit; Qiagen, Chatsworth, CA, USA). Insert analysis was carried out by *Eco*RI and *Not*I digestion and electrophoretic separation on 1% agarose gels.

2.2. cDNA sequencing and subcloning

Double-stranded DNA of positive clones was first sequenced in both directions according to the dideoxy method of Sanger et al. [14] with a T7 Sequencing Kit (Pharmacia LKB Biotechnology, Sweden) using universal and reverse primers, both commercially available. Among recombinant clones, pPHLP5B1912 was then identified by comparing its deduced amino acid sequence at the 5′ end of the cDNA sequence with the previously sequenced N-terminus of native *Phl p* Vb [15]. To determine the complete cDNA sequence, pPHLP5B1912 was restricted either with *Pst*I, *Xho*I or *Sal*I and fragments were sub-cloned into pBluescript plasmid for further nucleotide sequencing.

2.3. Purification recombinant Phl p Vb

Mature recombinant *Phl p* Vb was constructed by PCR amplification using pPHLP5B1912 as a template and two sequence-specific primers (5′-CGCTCATATACCGCGGATCCC and 3′-ATATAAGCTTTCC-TCTGAAGGAAGGCAACCC), by performing 30 cycles with 30 s at 95°C, 30 s at 53°C, 1.5 min at 72°C. The PCR product was purified from 1.2% agarose gels, restricted with *Bam*HI and *Hin*dIII and ligated into the expression vector pQE9 (Qiagen, Chatsworth, CA, USA). The correct reading frame was controlled by sequencing, and the recombinant protein was purified on a Ni^{2+}-NTA-resin column according to the instructions of the manufacturer (Qiagen).

2.4. Gel-filtration of Phl p Vb

Phl p Vb was further purified using gel-filtration on a Superdex 7S HR 10/30 column (Pharmacia LKB Biotechnology, Sweden) at room temperature. A flow rate of 0.6 ml/min of 50 mM sodium phosphate, 150 mM sodium chloride and 200 mM arginine, pH 6.0, eluted *Phl p* Vb at approximately 15 min. 20 s fractions were collected over the peak and pooled as appropriate. UV detection was done at 280 nm on an HPLC system (Kontron Instruments, Watford, UK) with a fraction collector.

2.5. Purification of natural Phl p Va

Natural *Phl p* Va was purified by affinity chromatography using Mab BG6, specific for group Va grass pollen allergens, covalently bound to a HiTrap NHS-activated affinity column (Pharmacia Biotech, Sweden), according to the description of the manufacturer.

*Corresponding author. Fax: (49) (4537) 10404.

SSDI 0014-5793(95)00259-6

2.6. Isolation of RNA

We extracted RNA from 5 g timothy grass pollen or 2 g inflorescence by a slightly modified method of Walsh et al. [16]. Pollen or inflorescence were ground in liquid nitrogen and broken with a pestle and mortar, suspended in 50 ml extraction buffer (400 mM NaCl, 50 mM Tris-HCl, pH 9, 5 mM EDTA, pH 8, 1% SDS, 10 mM DTT, 10 mM heparin, 1 mM aurinotricarboxylic acid) and 25 ml phenol/chlorophorm/isoamylalcohol (PCI; 24:24:1) followed by homogenization with an Ultra-Turrax (Janke & Kunkel, Ika-Werk, Hamburg, Germany) and centrifugation. The supernatant was extracted twice with PCI, and twice with chloroform/isoamylalcohol (24:1). RNA was precipitated with 2 M LiCl (final concentration). Purification of mRNA was done as described earlier [9].

2.7. RNA degradation on agarose gels

Ribonuclease A (RNase A, DNase free) and human placental ribonuclease inhibitor (RNasin) were purchased from Boehringer (Mannheim, Germany); RNA markers were from Gibco-BRL (Gaithersburg, USA). 2 μg of RNA or 1 μg of mRNA were incubated in RNase-free water with different substances for 45 min at the appropriate temperature. The mixture was then separated on a 1.2% agarose gel stained with 0.003% ethidium bromide.

2.8. RNase activity gel

RNase activity gel was done according to Yen and Green [17], performing SDS-PAGE containing 2 mg/ml torula yeast RNA (Sigma Chemical Co., St. Louis, MO, USA). After electrophoresis the gel was washed with 25% isopropanol in 0.01 M Tris-HCl to remove the SDS,

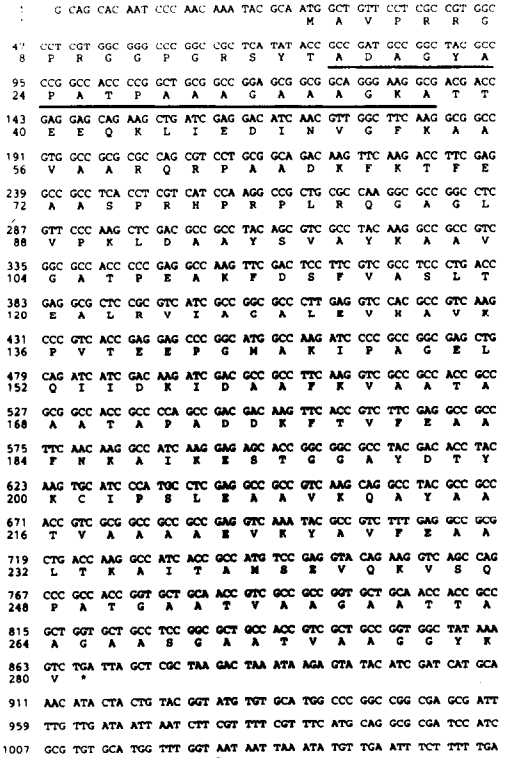

Fig. 1. Nucleotide and deduced amino acid sequence of the cDNA insert of pPHLP5B1912. Numbers indicate nucleotides (upper line) and amino acids (lower line). The N-terminal sequence 100% homologous to native *Phl p* Vb is underlined (sequenced by Edman degradation [15]). The broken line indicates a possible polyadenylation signal.

Fig. 2. Deduced amino acid sequence alignment of *Phl p* Vb with six different group V isoforms which belong to four different grass pollen species. PHOI_L_V from *Holcus lanatus* (Schramm et al. (1993) EMBL Nucleotide Sequence Data Library, Z27083); PLOLPIX from *Lolium perenne* [3]; PPOAP_IX._41 and 60 from *Poa pratense* [7]; PPHLPVA from *Phleum pratense* [9]; PPHLPV from *Phleum pratense* [8]. Numbers indicate amino acid residues. Hatched boxes show identity and homology, lines (-) are gaps in the sequences produced by the aligning program.

preincubated in 0.1 M Tris-HCl, incubated in 0.1 M Tris-HCl for 45 min at 56°C, and afterwards stained with Toluidine blue (Sigma; 0.2% in 0.01 M Tris-HCl) and finally destained in 0.01 M Tris-HCl.

2.9. RNase activity assay

RNase activity was determined according to Barna et al. [18] with modifications. Proteins were incubated with 1 μg timothy grass RNA. After incubation, 1 vol. 4 M LiCl was added to the mixture. It was stored at 4°C for at least 3 h and then centrifuged at 12,000 × *g* for 12 min. The supernatant was diluted and absorption was measured at 260 nm. One unit was defined as the amount of protein causing an increase in absorption of 1.0 after 30 min incubation at 56°C at pH 6.5.

2.10. Protein extraction from pollen, SDS-PAGE and Western blot

Proteins were extracted from 1 g timothy grass (Artu Biologicals N.V., Lelystad, The Netherlands). Pollen was incubated in 55 ml of 0.1 M NH_4HCO_3 buffer (pH 8) for 30 min at room temperature. After centrifugation for 30 min at 17,000 × *g* (4°C), the supernatant was dialyzed against double-distilled water, lyophilized and dissolved in appropriate sample buffer for SDS-PAGE. In a further procedure the extracted pollen proteins were applied to an RNase activity gel this time using a gradient SDS-PAGE according to Neville [19]. Regions showing RNase activity were excised from the gel and gel pieces were applied to a vertical 12% SDS-PAGE [21]. After electrophoresis proteins were

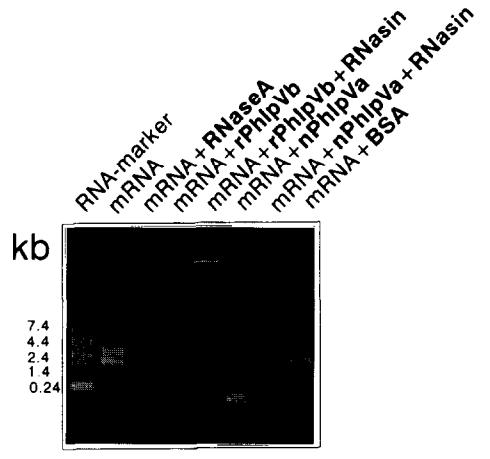

Fig. 3. Analysis of RNase activity of recombinant *Phl p* Vb and natural *Phl p* Va in a 1.2% agarose gel with 2 μg mRNA of timothy grass inflorescence, stained with ethidium bromide after incubation (45 min at 56°C) with H_2O, 2 μg RNase A, 2 μg recombinant *Phl p* Vb alone and with 32 U RNasin, 2 μg natural *Phl p* Va alone and with 32 U RNasin, or 2 μg bovine serum albumin (BSA).

electroblotted onto nitrocellulose membrane by a Fastblot apparatus (Biometra, Göttingen, Germany) and stained with antibodies as previously described [6] and finally cut in strips. The strips were stained with India ink and auro dye. Immunostaining was performed with Mabs Bo1, BG6, Bo9, all specific for group V allergens in timothy grass, and with patients' serum in a procedure described earlier.

2.11. Induction of pollen and inflorescence proteins by salicylic acid (SA)

Timothy grass inflorescence was harvested from wild plants 10 to 2 days before anthesis, grassheads cut from the stem and immediately ground in liquid nitrogen and stored at −80°C until further use. Induction of PR proteins was done according to Yalpani et al. [20]. In brief, 5 mg pollen or 2 g of grassheads were incubated at room temperature for a specified time with 500 μl or 30 ml 10 mM potassium phosphate buffer (pH 6.5) containing 1 mM EDTA and specified concentrations of SA. After centrifugation for 30 m in at 17,000 × *g* (4°C), the supernatant was dialyzed against double-distilled water, lyophilized and dissolved in appropriate sample buffer for SDS-PAGE. SDS-PAGE was done according to Laemmli [21], followed Western blot and immunological detection as described above.

2.12. Computer analysis

Sequence data were analyzed on GeneWorks and PCGENE Software (Intelligenetics, Geel, Belgium) and on DNASIS Software (Pharmacia LKB Biotechnology, Sweden).

2.13. Nucleotide sequence accession number

The nucleotide sequence of pPHLP5B1912 has been submitted to the EMBL Nucleotide Sequence Data Library and assigned accession number Z27083.

3. Results

3.1. cDNA and deduced amino acid sequence of Phl p *Vb*

The recombinant clone pPHLP5B1912 was isolated by immunoscreening of a timothy grass pollen cDNA library with monoclonal antibody (Mab) Bo1. Fig. 1 shows the nucleotide and deduced amino acid sequence of the identified open reading frame of 840 bp predicting a molecular weight of 28,142 Da. A possible polyadenylation site is indicated. pPHLP5B1912 was found to code for *Phl p* Vb by a 100% identity of the first 20 N-terminal amino acids (Fig. 1, underlined) to the sequence of natural *Phl p* Vb previously sequenced by Edman degradation [15]. Additionally, the fusion protein of pPHLP5B1912 is recognized by patients' IgE antibodies, Mab Bo1 and Mab Bo9 (data not shown) of which Bo9 is known to be specific for the acidic isoform *Phl p* Vb [13]. The putative leader peptide of 17 amino acids is 60% identical to the signal peptide of *Lol p* IX [3], a group V allergen of rye grass, which was shown to be targeted to the amyloplast in pollen.

3.2. Homology of Phl p *Vb to other group V allergens*

The homology among group V grass pollen allergens sequenced so far is 75–92%. Fig. 2 shows an alignment of seven different group V isoforms which belong to four different grass pollen species. The sequences spanning the central region between aa 68 and 239 of *Phl p* Vb determined by us are highly constant within the different proteins. The two amino acids at position 127 (alanine) and 128 (lysine) are missing in the four basic isoforms (*Phl p* V, *Phl p* Va, *Poa p* IX/41 and *Poa p* IX/60). This is the region of the Mab BG6 epitope which only recog-

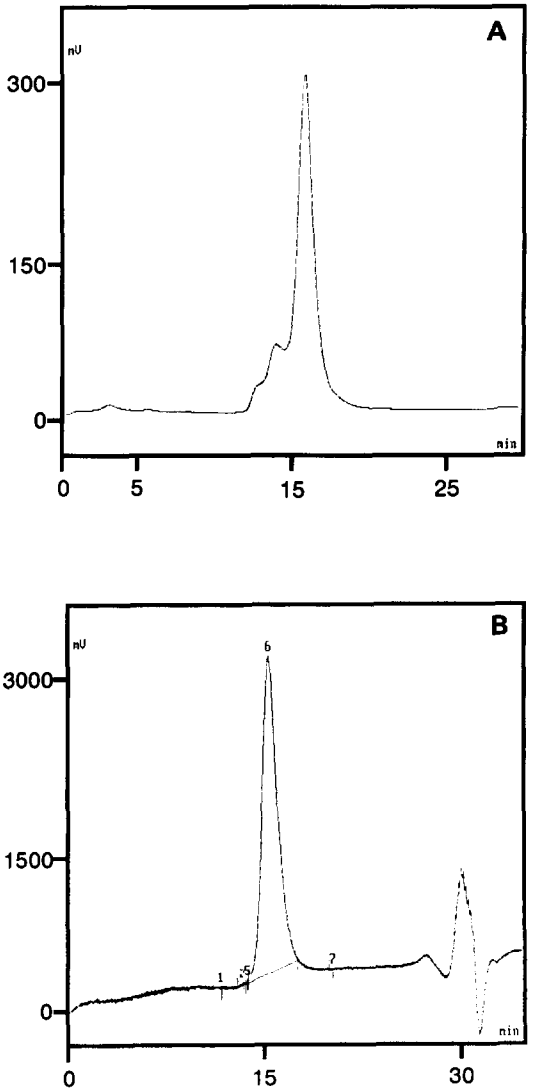

Fig. 4. Analytical HPLC flow sheet of Ni-chelate purified *Phl p* Vb (N) after elution (A) and HPLC purified *Phl p* Vb (P) after collection, pooling and concentration of fractions 8–12 over the peak (B). mV, voltage; min, elution time.

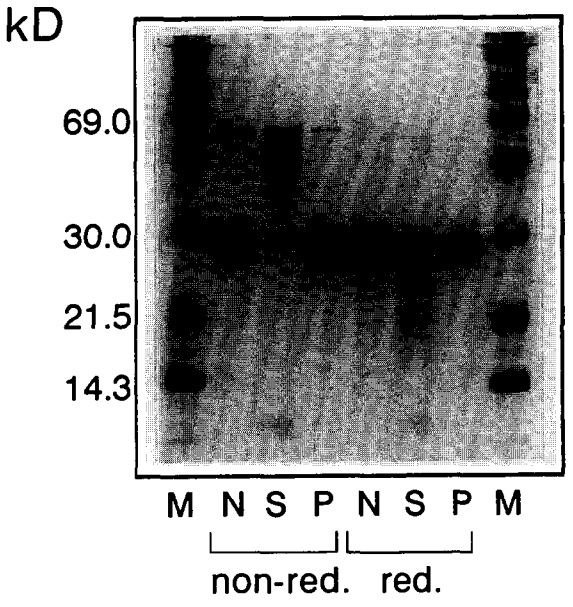

Fig. 5. 12% SDS-PAGE of the Ni-chelate fraction (N), the shoulder fraction (S) and the HPLC purified fractions 8–12 (P) of recombinant *Phl p* Vb. non-red., non reduced; red., reduced with 3% β-mercaptoethanol. M, marker.

nizes *Phl p* Va but not *Phl p* Vb (data not shown). The N- and C-terminal part of group V allergens exhibit the variable domain mainly characterized by genetic deletions rather than by single amino acid exchanges.

3.3. *Ribonuclease activity of* Phl p *Vb*

Recombinant *Phl p* Vb, which was cloned into the expression vector pQE expressed as a six histidine non-fusion protein, was purified via a Ni-chelate column. The purified allergen was incubated with timothy grass mRNA at 56°C for 45 min. The results in Fig. 3 show that mRNA was completely degraded by purified recombinant *Phl p* Vb, an action which was inhibited by the RNase inhibitor RNasin, clearly visible as a complex of mRNA and the allergen at the top of the gel. In a gel-shift experiment it was revealed that recombinant *Phl p* Vb and mRNA form a complex which, compared to *Phl p* Vb alone, has a reduced mobility in native polyacrylamide gels (data not shown). To exclude the possibility of contamination with other RNases, further purification of the recombinant protein was performed. The HPLC flow sheet of the Ni-chelate purified *Phl p* Vb in Fig. 4A shows the protein peak eluting after about 15 min partly mixed with additional products indicated by a peak shoulder appearing just ahead the major peak. 20 fractions over the major peak were collected. Fractions 8–12 contained the completely pure *Phl p* Vb, as shown in Fig. 4B. The SDS-PAGE gel (Fig. 5) of the non-reduced and reduced Ni-chelate purified *Phl p* Vb (N), the HPLC peak shoulder fraction (S), and the completely purified *Phl p* Vb (P) additionally demonstrates the purity of the recombinant protein. It also shows that S seems to consist of aggregates which obviously bind to native soluble recombinant *Phl p* Vb. In a Western blot of this SDS-PAGE gel (data not shown) the shoulder peak aggregates mainly bound to a group V Mab. We suggest that these aggregates are in part so called 'early quitters', that is, not fully translated recombinant *Phl p* Vb fragments.

Fractions N, S and P were applied to an RNase agarose gel (Fig. 6). N and P degrade RNA nearly completely but also show complexation with RNA. S has no RNase activity but seems to complex slightly, indicating that it still contains small amounts of complete *Phl p* Vb.

3.4. *Ribonuclease activity of natural* Phl p *V*

Affinity purified natural *Phl p* Va degraded the mRNA only partly, the activity being inhibited by RNasin (Fig. 3). No complexation of *Phl p* Va and RNA was observed. It is possible that the allergen could have lost its activity during the purification process. To analyze whether just extracted pollen allergens show RNase activity we isolated proteins from timothy grass pollen and applied them to an RNase activity gel. Fig. 7 demonstrates that pollen extract contains at least four different RNase bands. In order to determine whether the bands at 38 and 32 kDa represent group V allergens these RNase active bands were excised from the gel and reseparated on an SDS-PAGE gel for further analysis in Western blot using different group V specific Mabs.

The results show that the RNase active band at 38 kDa, which was excised from the gel and stained by India ink and auro dye, consists of two proteins. The upper band at 38 kDa is recognized by *Phl p* Va specific Mabs and runs at the same molecular weight as the allergens in the timothy grass pollen extract. The lower band at 38 kDa also runs in the range of group Va, but is only bound by patients' IgE antibodies, indicating that it is an allergen but probably does not belong to the group V allergens. The RNase active band at 32 kDa is recognized by *Phl p* Vb specific Mabs but again there is a smear in a slightly lower kDa range bound by patient's IgE antibodies. Additionally, as is also seen with the recombinant protein (Fig. 6), natural *Phl p* Vb tends to form dimers and higher aggregates. The immunoreactivities of the excised RNases are weaker than that of the original proteins in the pollen extract

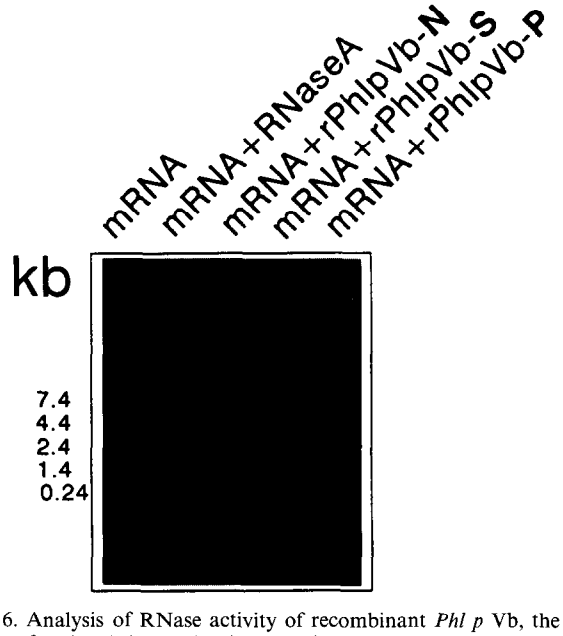

Fig. 6. Analysis of RNase activity of recombinant *Phl p* Vb, the Ni-chelate fraction (N), the shoulder fraction (S), and the HPLC purified fraction (P) on a 1.2% agarose gel with 2 μg mRNA of timothy grass inflorescence, stained with ethidium bromide.

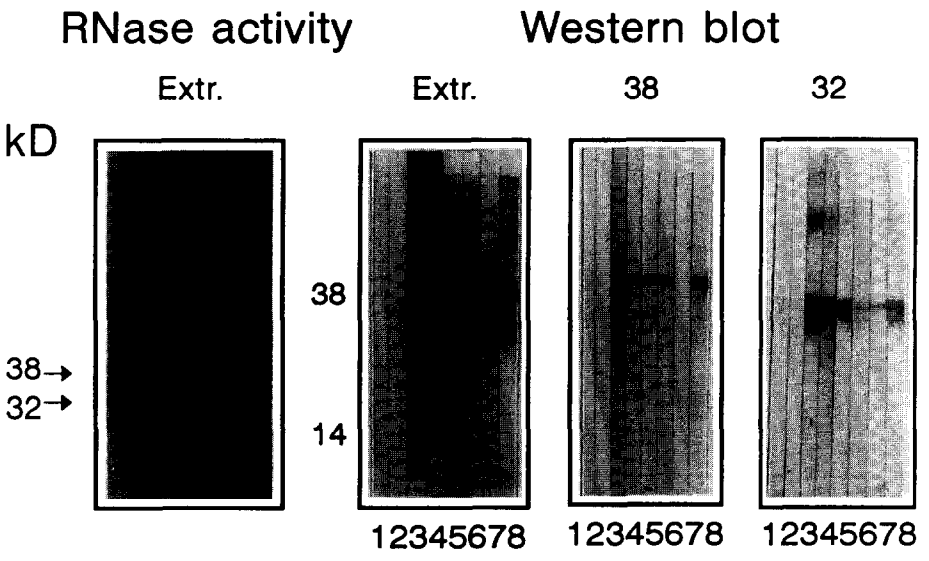

Fig. 7. RNase activity gel of timothy grass pollen extract (Extr.) and Western blot of pollen extract (Extr.), the 38 kDa RNase active band (38) and the 32 kDa RNase active band (32), stained with India ink (lane 3), auro dye (lane 4), Mab Bo1 (lane 5), Mab BG6 (lane 6), Mab Bo9 (lane 7) and patients' serum (lane 8). Lanes 1 (anti-IgG and IgM) and 2 (anti-IgE) are controls of the second AP conjugated antibodies.

which can be explained by the small amount of protein which is excised from the gel.

3.5. *Quantification of RNase activity*

The RNase activity of timothy grass pollen extract is 3030 U/mg and is thus 1000 times lower than pancreatic ribonuclease A as shown in Fig. 8. Only parts of it can be attributed to the group V allergens as the pollen contains additional RNases demonstrated in Fig. 7. Recombinant *Phl p* Vb has an activity of 830 U/mg and makes up about 25% of the whole extract activity. Whether this is comparable to the activity of the natural allergens remains unknown. In summary the specific activity of recombinant *Phl p* Vb is low compared to other known RNases.

3.6. *Induction of* Phl p *Vb in pollen and inflorescence*

RNases in plants can be involved in plant resistance processes. We used the plant hormone salicylic acid (SA), involved in induction of plant resistance genes [22,23]; incubation of timothy grass pollen and timothy grass inflorescence with increasing concentrations of SA was carried out over a period of 16 h. In grass pollen it did not lead to an additional release of group V allergens, as determined by Western blot with the anti-group V Mab Bo1 (Fig. 9A). In contrast, induction of timothy grass inflorescence (Fig. 9B) led to the release of a protein at 32 kDa, which was recognized by the group V specific Mab. The group V specific protein exuded from the plant had its maximum at a concentration of 10 μg/ml SA. The release was reduced again at 20 μg/ml SA. The 32 kDa protein in the 10 μg/ml SA fraction showed immunological reactivity with allergic patients serum IgE, but not with group Va Mabs and only faintly with group Vb Mabs (data not shown).

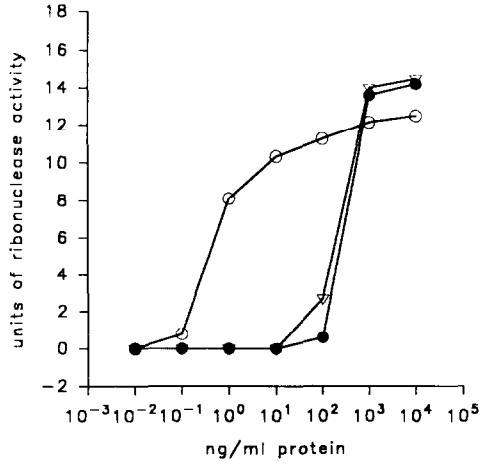

Fig. 8. RNase activity of recombinant *Phl p* Vb (filled circles) and natural timothy grass pollen extract (open triangles) compared with pancreatic RNase A (open circles).

4. Discussion

In this paper we present the cDNA clone coding for *Phl p* Vb, the acidic isoform of group V allergens in timothy grass pollen. Its relationship to this group of major allergens was defined by immunological reactivities with Mabs and by N-terminal microsequencing [5,15]. By comparing cDNA sequences of a number of group V clones it was confirmed that the heterogeneity is caused by genetic variations, a feature possibly reflecting the character of gene families. Similar observations have been made with major tree pollen allergens. A substantial number of isoforms of the major birch pollen allergen *Bet v* I were isolated by PCR technology which showed sequence variabilities influencing the immunoreactivity of the different isoforms [22]. The variable regions of group V grass pollen allergens are located at the N- and C-terminal parts of the molecules. These variable regions were found to bear IgE reactive epitopes in *Phl p* Va [9]. The existence of variable immunogenic structures may explain why patients' IgE reactivities to isoforms of group V allergens may be individually different.

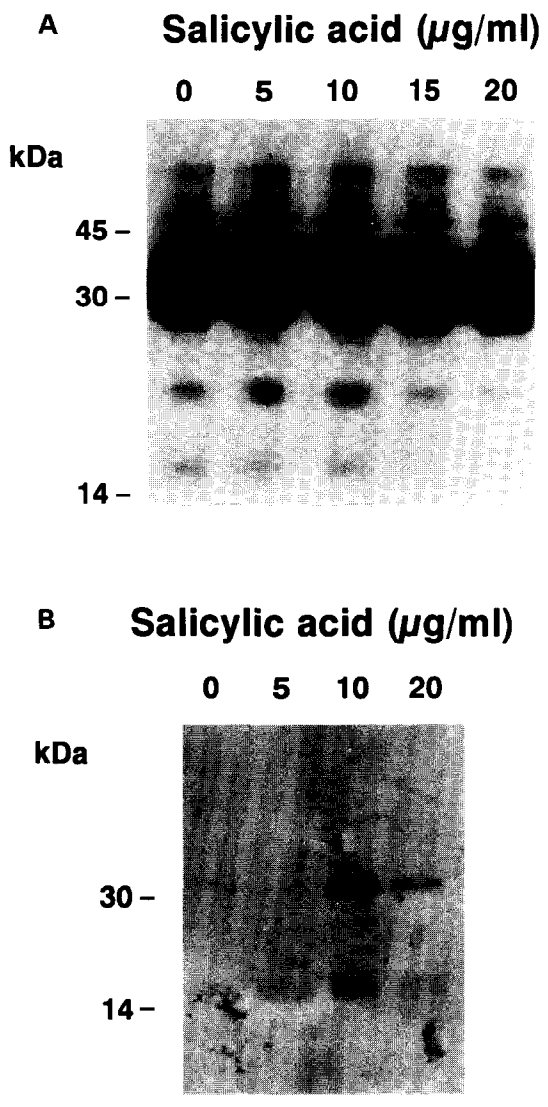

Fig. 9. Western blot of timothy grass pollen extract (A) and timothy grass inflorescence supernatant (B) after 16 h of incubation with different SA concentrations probed with Mab Bol.

The function of the group V allergens in grass pollen had not been elucidated until now. Sequence identity and homology of *Phl p* Vb to other proteins of known function were not found. We here present data that a protein released from the mother plant of timothy grass pollen after stimulation with a salicylic acid, a signal hormone of plant resistance [23,24], is highly cross-reactive with the group V pollen allergens. This strongly suggests that a group V allergen equivalent could be involved in host–pathogen interactions, although we did not demonstrate direct pathogen induced activation. Hodgkin et al. [25] suggested that some host pathogenically induced proteins may be evolutionarily and functionally related to plant RNases. This led us to examine whether group V allergens in grass pollen have ribonuclease activity. Indeed our experiments suggest that group V allergens of timothy grass pollen, and possibly homologous grass pollen allergens, are RNases, or at least show RNase activity. It is obvious that there exist a number of RNases in the pollen extract, of which the group V allergens are only one. Concerning the natural *Phl p* Va and b, it is still tempting to doubt the ribonuclease function. The activity seems to be rather weak in our system although there are two lines of evidence; (i) the activity of the affinity purified *Phl p* Va and (ii) the excised band from the yeast RNA-containing SDS-PAGE gel which showed immunological reactivity with a group V specific Mab. With the highly purified recombinant *Phl p* Vb protein we present clear evidence for RNase activity of a group V allergen. The activity of the recombinant version is low compared to known RNases. This fact could have been influenced by either the purification procedure, the refolding conditions of the recombinant protein, or perhaps unknown post-translational differences between the bacterial and the natural protein. On the other hand comparing the RNase activity of the complete natural pollen extract with the recombinant *Phl p* Vb shows the activity of the recombinant protein to be 25% of the natural extract. This might reflect the approximate quantitative relationship between *Phl p* Vb and the other RNases in the pollen extract, as shown in the RNase activity gel using yeast RNA. It could also indicate the different specificities of the pollen RNases.

It remains an open question what physiological function the pollen RNases may have. As we have seen, the group V allergens seem to be similar enough among different species in certain sites on the molecule to show a degree of immunological identity, but they are sufficiently diverse in other sites. This may reflect the genotypes of the plants concerned, a fact leading Knox et al. to the hypothesis that these proteins may be involved in recognition processes between the different species [10]. Only recently it was demonstrated that recognition substances can be proteins with ribonuclease activity [26], like S-locus RNases in tobacco plants which induce self-incompatibility (SI). Yet these SI proteins are part of the plant stigma whereas group V allergens are part of the pollen. We can only add to speculation about the role of the pollen RNases in grasses.

Further research has to reveal the physiological function of these RNases in the pollen–stigma interaction, and elucidate whether this function in itself influences the induction of allergy in humans.

Acknowledgements: We want to thank Daniela Warneke for her excellent technical assistance. This work was supported by a grant from the Forschungsinstitut Borstel to A.B. in the context of a two year molecular biology course at Hamburg University, conducted by Prof. H.H. Arnold, now of Braunschweig, Germany.

References

[1] Barnes, P.J. (1991) Trends Biochem. Sci. 16, 365–369.
[2] Knox, R.B. (1993) Clin. Exp. Allergy 23, 354–359.
[3] Singh, M.B., Hough, T., Theerakulpisut, P., Avjioglu, A., Davies, S., Smith, P.M., Taylor, P., Simpson, R.J., Ward, L.D., McCluskey, J., Puy, R. and Knox, R.B. (1991) Proc. Natl. Acad. Sci. USA 88, 1384–1388.
[4] Suphioglu, C., Singh, M.B., Taylor, P., Bellomo, R., Holmes, P., Puy, R. and Knox, R.B. (1992) Lancet 339, 569–572.
[5] Matthiesen, F. and Løwenstein, H. (1991) Clin. Exp. Allergy 21, 309–320.
[6] Petersen, A., Becker, W.-M. and Schlaak, M. (1992) Electrophoresis 13, 736–739.
[7] Silvanovich, A., Astwood, J., Zhang, L., Olsen, E., Kisik, F., Sehon, A., Mohapatra, S. and Hill, R. (1991) J. Biol. Chem. 260, 1204–1208.

[8] Vrtala, S., Sperr, W.R., Reimitzer, I., van Ree, R., Laffer, S., Müller, W.-D., Valent, P., Lechner, K., Rumpold, H., Kraft, D., Scheiner, O. and Valenta, R. (1993) J. Immunol. 151, 4773–4781.
[9] Bufe, A., Becker, W.-M., Schramm, G., Petersen, A., Mamat, U. and Schlaak, M. (1994) J. Allergy Clin. Immunol. 94, 173–181.
[10] Knox, R.B. (1973) J. Cell. Sci. 12, 421–443.
[11] Preuss, D., Lemieux, B., Yen, G. and Davis, R.W. (1993) Genes Dev. 7, 974–985.
[12] Becker, W.-M., Schaubschläger, W., Westphal, W. and Schlaak, M. (1988) in: Biochemical Engineering (Chmiel, H., Hammes, W. and Baily, J.E. eds.) pp. 440–462, Gustav Fischer Verlag, Stuttgart.
[13] Petersen, A., Becker, W.-M. and Schlaak, M. (1994) Clin. Exp. Allergy 24, 250–256.
[14] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463.
[15] Petersen, A., Schramm, G., Becker, W.-M. and Schlaak, M. (1993) Biol. Chem. Hoppe-Seyler 374, 855–861.
[16] Walsh, D.J., Matthews, J.A., Denmeade, R. and Walker, M.R. (1989) Int. Arch. Allergy Immunol. 90, 78–83.
[17] Yen, Y. and Green, P.J. (1991) Plant Physiol. 97, 1487–1493.
[18] Barna, B., Ibenthal, W.D. and Heitefuss, R. (1989) Physiol. Mol. Plant Path. 35, 151–160.
[19] Neville, D.M. and Glossmann, H. (1974) Methods Enzymol. 32, 92–102.
[20] Yalpani, N., Silverman, P., Wilson, T.M.A., Kleier, D.A. and Raskin, I. (1991) Plant Cell 3, 809–818.
[21] Laemmli, U.K. (1970) Nature 227, 680–685.
[22] Ipsen, H., Schou, C. and Klysner, S. (1993) in: Molecular Biology and Immunology of Allergens (Kraft, D. and Sehon, A. eds.) pp. 219–222, CRC Press, Boca Raton.
[23] Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993) Science 261, 754–756.
[24] Van de Rhee, M.D., Van Kan, J.A.L., Gonzalez-Jean, M.T. and Bol, J.F. (1990) Plant Cell 2, 357–366.
[25] Hodgkin, T., Lyon, G.D. and Dickenson, H.G. (1988) 110, 557–569.
[26] Murfett, J., Atherton, T.L., Mou, B., Gasser, C.S. and McClure, B.A. (1994) Nature 367, 563–566.