Research

Mass spectrometric analysis of electrophoretically separated allergens and proteases in grass pollen diffusates

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Abstract

Background: Pollens are important triggers for allergic asthma and seasonal rhinitis, and proteases released by major allergenic pollens can injure airway epithelial cells *in vitro*. Disruption of mucosal epithelial integrity by proteases released by inhaled pollens could promote allergic sensitisation.

Methods: Pollen diffusates from Kentucky blue grass (*Poa pratensis*), rye grass (*Lolium perenne*) and Bermuda grass (*Cynodon dactylon*) were assessed for peptidase activity using a fluorogenic substrate, as well as by gelatin zymography. Following one- or two-dimensional gel electrophoresis, Coomassie-stained individual bands/spots were excised, subjected to tryptic digestion and analysed by mass spectrometry, either MALDI reflectron TOF or microcapillary liquid chromatography MS-MS. Database searches were used to identify allergens and other plant proteins in pollen diffusates.

Results: All pollen diffusates tested exhibited peptidase activity. Gelatin zymography revealed high M_r proteolytic activity at ~ 95,000 in all diffusates and additional proteolytic bands in rye and Bermuda grass diffusates, which appeared to be serine proteases on the basis of inhibition studies. A proteolytic band at M_r ~ 35,000 in Bermuda grass diffusate, which corresponded to an intense band detected by Western blotting using a monoclonal antibody to the timothy grass (*Phleum pratense*) group 1 allergen Phl p 1, was identified by mass spectrometric analysis as the group 1 allergen Cyn d 1. Two-dimensional analysis similarly demonstrated proteolytic activity corresponding to protein spots identified as Cyn d 1.

Conclusion: One- and two-dimensional electrophoretic separation, combined with analysis by mass spectrometry, is useful for rapid determination of the identities of pollen proteins. A component of the proteolytic activity in Bermuda grass diffusate is likely to be related to the allergen Cyn d 1.

Introduction

In most economically-developed countries, at least 20% of the population suffers from IgE-mediated (Type I) allergic diseases, typically manifesting as asthma or seasonal rhinitis/conjunctivitis. In the majority of patients, this is related to sensitisation to airborne allergens [1,2] but the mechanisms by which exposure triggers an allergic response remain incompletely understood. Nor is it clear why the incidence of allergic asthma and rhinitis in economically-developed countries, including Australia, is so

high. Although genetic and environmental factors both influence induction of an IgE response to an inhaled antigen [3], environmental exposure to allergens is likely to be particularly important; for example, the prevalence of asthma and rhinitis in immigrants to Australia increases significantly with the length of stay [4].

Tight junctions between airway epithelial cells apparently constitute a physical barrier between inhaled antigens and the immune system. If the access of allergenic proteins to the subepithelial antigen-presenting dendritic cells is facilitated as a consequence of breaching the integrity of the airway epithelial barrier at the time of initial exposure, for example by concurrent infection or exposure to environmental pollutants, an IgE response may be stimulated [5]. In this context, it is noteworthy that several important allergens of the house dust mite [6,7], cat allergen [8] and some fungal allergens [9] have intrinsic protease activity. Exposure to the serine protease major mite allergen *Der p 1* disrupts epithelial tight junctions, increases epithelial permeability and may facilitate allergen delivery [10–12]. Experimental studies *in vivo* suggest that the protease activity of *Der p 1* promotes induction of an IgE response, to both *Der p 1* and bystander antigens [13,14].

Airborne allergens derived from plant pollens are important triggers for asthma and rhinitis. Pollens do contain a variety of enzymes, including proteases. Because 20–25% of the pollen mass is released rapidly on hydration [15], very high concentrations of pollen solutes are likely to be achieved when pollen grains are deposited on mucosal surfaces. However, there is little information about whether allergenic proteins of pollens have intrinsic protease activity [16]. Proteases from a limited number of allergenic pollens have been purified (e.g. [17–19]), but to date there is only one set of reports suggesting that a pollen allergen might have protease activity [20,21].

We previously showed that proteases released by a variety of allergenic pollens cause concentration-dependent detachment of airway epithelial cells in culture [22]. Significantly, these proteases were not efficiently blocked by major endogenous antiproteases, including a1-proteinase inhibitor and secretory leucocyte protease inhibitor. Thus, it is plausible that disruption of the epithelial barrier by proteases in pollens might promote sensitisation, not only to the protease but also to simultaneously released pollen proteins with no intrinsic enzymatic activity. Our preliminary characterisation of the proteolytic activity in diffusates of various allergenic pollens revealed that some (e.g. Kentucky blue grass) exhibited high substrate preference for Arg and Lys while others (e.g. ryegrass, Bermuda grass, ragweed) cleaved a Cys-containing substrate most rapidly and were also associated with marked preference for hydrophobic amino acids Leu and Met. These patterns

were not mutually exclusive because Acacia pollen diffusate exhibited an overlapping profile of activities [23].

In the present study, we sought to directly test the relationship between pollen allergens and proteases. We employed a micro-plate assay using a fluorescent substrate, as well as gelatin zymography, to assess peptidase and protease activity present in pollen diffusates. Pollen diffusate proteins were separated by one- and two-dimensional SDS/PAGE. Proteolytically active bands/spots were then identified after "in gel" tryptic digestion, mass spectrometry and database searches.

Materials and Methods
General
Reagents and chemicals were analytical grade (Sigma, St. Louis, MO, USA; BioRad, Hercules, CA, USA) and solvents were HPLC grade (Mallinckrodt, Clayton South, Vic., Australia). SDS/PAGE/Western blotting were performed using a Mini Protean II apparatus (BioRad, Hercules, CA) with 12 or 15% gels and a Tris/Tricine buffer system [24]. Two-dimensional PAGE was performed using Immobiline IPG strips (7 cm) with a pH range of 3–10 (Amersham, Biosciences, UK). The monoclonal antibody known as IG 12, to the allergen Phl p 1 of timothy grass (*Phleum pratense*), was a gift from Drs Kay Grobe and Arnd Petersen [20]. Protein concentrations were determined using a micro BCA assay (Pierce, Rockford, IL). Amino acid sequences were compared using the best fit program in the Genetics Computer Group Package, Version 8, Madison, WI, USA http://www.angis.org.au.

Preparation of pollen diffusates
Pollens from Kentucky blue grass (*Poa pratensis*), rye grass (*Lolium perenne*) and Bermuda grass (*Cynodondactylon*) were purchased from Bayer Australia (Sydney, Australia). Diffusates of the pollens were prepared by thoroughly mixing dry pollen (200 mg) with normal saline (2 ml, pH ~ 7.5) and incubating without agitation at 37°C for 60 min [23]. The mixture was centrifuged at 16,000 g for 10 min and the pellet washed (1 × 500 μl) with saline. Supernatants were pooled, filtered (0.22 μm, Millex-GV, Millipore, Bedford MA) and total protein concentrations determined.

Proteolytic Activity towards a peptide substrate
Diffusates (4 μl, ~ 1 mg/ml) and trypsin (1 μg, Sigma) were incubated with Nα-benzoyl-L-Arg 7-amido-4-methylcoumarin (NBAMC, 4 μl, 1 mg/ml, Sigma) [25] and PBS (100 μl, 25 mM phosphate, 250 mM NaCl) in a 96 well plate (Nunc, Roskilde, Denmark) for 1 min, then fluorescence ($Ex_{360 > nm}$, $Em_{460 nm}$) measured at 1 min intervals for 60 min using a Cytofluor plate reader (Perspective Biosystems, Framingham CT). Protease inhibitors, phenylmethylsulfonyl fluoride (PMSF, 1 μl, 200 mM), complete

Table 1: Summary of pollen diffusate proteins identified by micro C18 RP-HPLC and ESI.

Spot	Gel (M$_r$)	micro LC-ESI	Mass (calc)
1	30,000	Acidic Cyn d 1 isoallergen isoform 3 precursor	28,378
2	30,000	Acidic Cyn d 1 isoallergen isoform 4 precursor	28,407
3	30,000	Acidic Cyn d 1 isoallergen isoform 2/4 precursor	28,391
4	30,000	Acidic Cyn d 1 isoallergen isoform 2/4 precursor	28,407
5	25,000	Acidic allergen Cyn d 1 precursor	28,391
6	25,000	Acidic allergen Cyn d 1 precursor	26,645
7	25,000	Acidic allergen Cyn d 1 precursor	26,645
8	35,000	Acidic allergen Cyn d 1 precursor	26,645
9	50,000	Acidic Cyn d 1 isoallergen isoform 1 precursor	26,663
10	50,000	Acidic Cyn d 1 isoallergen isoform 1 precursor	26,663
11	50,000	Phosphoglucomutase, cytoplasmic (PGM2)	63,002
12	35,000	Acidic Cyn d 1 isoallergen isoform 1 precursor	26,663
13	35,000	L-Ascorbate peroxidase, cytosolic isozyme, maize	27,295
14	35,000	Acidic Cyn d 1 isoallergen isoform 1 precursor	26,663
15	35,000	Phosphoglucomutase, cytoplasmic 2	63,002
16	40,000	Acidic Cyn d 1 isoallergen isoform 1 precursor	26,663
17	40,000	Triosephosphate isomerase, cytosolic (TIM)	27,008
18	40,000	Enolase 2	48,132
19	40,000	Profilin 3 (ZmPRO3)	14,228
20	50,000	Pistilata homolog ScPI (Sanguinaria Canadensis)	24,075
21	60,000	Acidic Cyn d 1 isoallergen isoform 1 precursor	26,663
22	70,000	Acidic allergen Cyn d 1 precursor	26,645
23	60,000	Acidic allergen Cyn d 1 precursor	26,645
24	60,000	Acidic Cyn d 1 isoallergen isoform 2 precursor	28,391
25	90,000	Acidic allergen Cyn d 1	26,645
26	12,000	Acidic allergen Cyn d 1	26,645
27	18,000	Acidic allergen Cyn d 1 precursor	26,645
28	50,000	Acidic allergen Cyn d 1 precursor	26,645
29	50,000	Acidic allergen Cyn d 1 precursor	26,645
30	50,000	Enolase	47,956

protease inhibitor cocktail (1 µl, diluted in H$_2$O to give a stock solution according to the manufacturers instructions, Roche), N$_\alpha$-Tosyl Phe chloromethane ketone (TPCK, 4 µl, 100 µM), Tosyl Lys chloromethyl ketone (TLCK, 4 µl, 100 µM), 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF, 1 µl, 4 mM) iodoacetamide (1 µl, 100 mM) or ethylenediaminetetraacetic acid (EDTA, 1 µl, 500 mM) were incubated with diffusates (4 µl) for 60 min prior to addition of substrate and fluorescence measurement.

SDS/PAGE and Zymography
Pollen diffusates (25 µl, ∼ 1 mg/ml) were treated with PMSF (3 µl, 300 mM) AEBSF (100 µl, 8 mM), complete protease inhibitor (1 µl of stock solution), iodoacetamide (10 µl, 100 mM) for 1 h before diluting with loading buffer (10 µl, BioRad) and separation by SDS/PAGE or zymography [26]. SDS/PAGE gels were stained with Coomassie brilliant blue (CBB, 0.4% R-250, Sigma), silver or transferred to PVDF membranes (Immobilon P, Millipore, MA, USA) before Western analysis and detection using enhanced chemiluminescence.

Zymograms were prepared using either gelatin (1%, Sigma) or casein (1%, Sigma) co-polymerised in 10 or 12.5% acrylamide according to published procedures [26]. After electrophoresis, gels were incubated in PBS (1% Triton X-100, 20 ml) for 60 min and TBS (20 ml) at 37°C for 14 hours, then stained with CBB (0.4%) in H$_2$O for 60 min. After destaining in methanol/H$_2$O/acetic acid (25:75:5 v/v) for 3 hours (∼ 4 × 30 ml), an image of the gel was obtained using a Gel Doc (BioRad).

Two-Dimensional SDS/PAGE and Zymography
To achieve satisfactory results, Bermuda grass pollen diffusate (250 µl, ∼ 100 mg/ml) was concentrated and desalted to 50 µl using a Centricon 10 kDa cut-off ultracentrifugation device (Millipore, USA). The concentrated diffusate was dissolved in 75 µl of IPG gel rehydration buffer containing 8 M urea, 2% w/v CHAPS, 2% carrier ampholytes (100 × Bio-lyte, pH range 3–10 Bio-Rad, USA) and traces of Bromophenol blue. Reducing agents such as DTT or tributyl phosphine were excluded from the rehydration buffer as they destroyed the proteolytic activity present in the diffusate. Separation of the proteins in the first phase

was followed by a short equilibration step of the IPG strip in SDS equilibration buffer, after which proteins were separated by SDS-PAGE and/or gelatin zymography as described above.

Peptide Mass Finger Printing

Stained bands from one-dimensional electrophoresis were excised and washed with NH_4HCO_3 (100 μl, 100 mM, 15 min), CH_3CN (150 μl, 10 min), NH_4HCO_3 (100 μl, 10 mM, 10 min), CH_3CN (150 μl, 20 min), then dried using a Speedvac (Savant, Farmingdale, NY) for 10 min. Gels pieces were rehydrated with NH_4HCO_3 (20 μl, 10 mM) containing either trypsin (250 ng) or AspN (100 ng) and incubated for 14 hours at 37°C. Digest buffer containing peptides (0.5 μl) was mixed with DHB matrix (1 μl, 10 mg/ml) allowed to air-dry and analysed by reflectron TOF mass spectrometry over a mass range of m/z 550 to 5,000. Approx. 250 spectra were acquired and averaged. Positive ions were generated using a N_2 laser (337 nm, 3-nsec pulse width) and accelerated to 25 keV, an extraction delay of 175 nsec (Voyager STR, Perspective Biosystems, Framingham, MA). Spectra were calibrated externally using the monoisotopic masses of angiotensin I and oxidised insulin B chain. Peptides masses were entered manually into the peptide mass fingerprinting search program Mascot http://www.matrixscience.com. Non-redundant protein databases ($NCBI_{nr}$) were searched and search results were tabulated and scores assigned allowing assignment of likely proteins.

Microcapillary liquid chromatography/MS-MS

Fused silica capillaries (200 μm × ~ 15 cm) were packed with an acetonitrile slurry of C18 resin (C18 Widepore, Bakerbond, Phillipsburg, NJ, USA) with a 1 cm piece of capillary (50 μm × 190 μm) preventing leaching of the resin from the outlet. Columns were coupled to a low volume stainless steel connector where HV (~ 2 kV) was applied and the outlet was connected to a piece of fused silica (~ 2 cm) that was pulled to a tip diameter of ~ 25 μm. The tip was positioned ~ 2–3 mm from the heated capillary (175°C) of a TSQ 7000 MS (Finnigan, San Jose CA). An HP1090 LC system (Hewlett Packard, San Jose, CA) forming a gradient of 100% H_2O (0.1% formic acid) to 60% CH_3CN (0.1% formic acid) over 40 min was applied at a flow rate of 100 μl/min which was split 1:100, such that the flow from the column was ~ 1 μl/min. Peptide solutions from gel digests (up to 5 μl) in formic acid (1%) were injected manually using a Rheodyne 8125 injector (5 μl loop, Rheodyne, CA, USA). Electrospray ionisation mass spectra were acquired from m/z 400 to 1600 in 1 S. The most intense ion from each spectrum, that exceeded a preset threshold, was automatically selected for low energy collision induced dissociation (CID) MS/MS analysis with a collision energy of 23 V and collision gas (Ar) at a manifold pressure of ~ 1.2 Torr [27]. The

identities of peptides were confirmed by searching amino acid sequence databases (SwissProt) with tandem mass spectra using the SEQUEST algorithm (ThermoFinnigan, San Jose, CA) or MS/MS search program from Matrix science (Mascot, http://www.matrixscience.com) using the $NCBI_{nr}$ database. Search results were tabulated and scores assigned allowing identification of proteins in the digests.

Results and Discussion

Determination of Diffusate Peptidase Activity for a Peptide Substrate

Diffusates from grass pollens were assayed for peptidase activity with NBAMC, a fluorogenic substrate with tryptic specificity. Profiles typical of fluorescence vs time for Kentucky blue diffusate and trypsin after incubation for 1 hour are shown in Fig. 1A. All diffusates digested the substrate immediately upon addition of the diffusate and gave similar results. Using the same diffusate protein concentration, the relative proteolytic activity of rye grass diffusate was comparable to that of trypsin after 1 hour of incubation, whereas the activity of Kentucky blue grass and Bermuda grass diffusates was approximately 75% less. The fluorescence of each diffusate, reached after 1 hour, was scaled to 100 as were changes in activity observed after addition of an inhibitor (Fig 1B). This approach allows ready comparison of activities and similar results were obtained in repeated experiments (n = 3, not shown). All proteolytic activity was almost completely eliminated by the complete protease inhibitor cocktail or the effective serine protease inhibitor AEBSF. Trypsin-like activity was confirmed by the almost complete inhibition with TLCK, whereas inhibition by TPCK, a chymotrypsin-specific inhibitor, was very modest (Fig 1). Little inhibition was observed with the cysteine protease inhibitor iodoacetamide on Bermuda grass diffusate, whereas the activity of Kentucky and rye grasses was inhibited by ~ 40%, suggesting that these diffusates contain cysteine peptidase activity. The cysteine protease papain was completely inhibited by iodoacetamide (not shown). The metalloprotease inhibitor EDTA was inactive.

These results indicate that, under the conditions of assay, the predominant peptidase activity for the substrate NBAMC present in these pollen diffusates is due to serine-type enzymes, but cysteine protease activity was also detected in Kentucky and rye grass diffusates. The total fluorescence levels observed after 1 hour incubation varied by only ~ 75% between the pollen diffusates (not shown). It was not possible to determine relative levels of proteases present in the diffusates from these experiments. These studies indicate that all the pollen diffusates examined here possessed intrinsic activity for Lys/Arg substrates. However, whereas our previous study had suggested that trypsin-like peptidase activity was a minor component of the activity in rye and Bermuda grass and
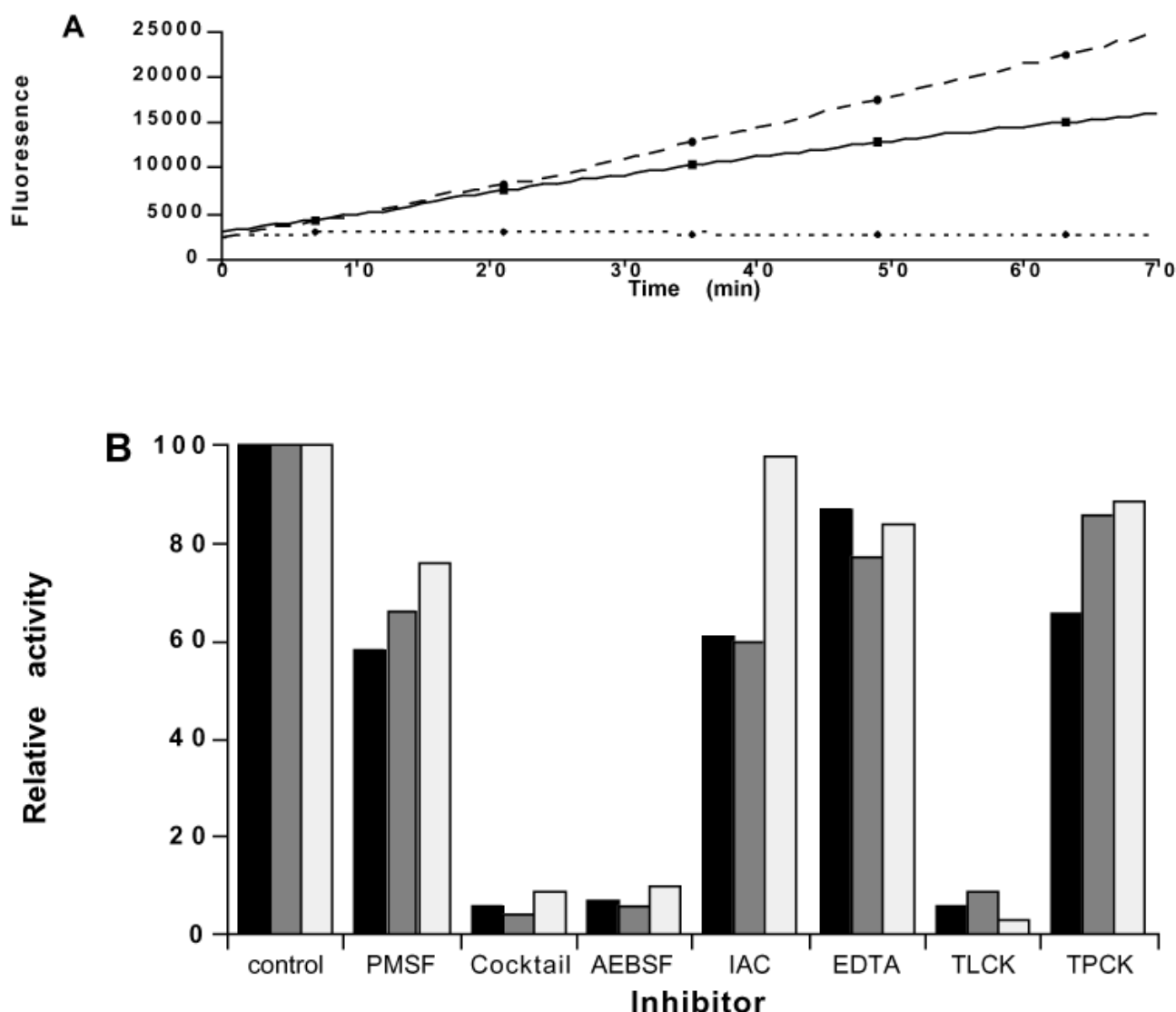
Figure 1
Comparison of proteolytic activity towards a peptide substrate in grass pollen diffusates. A. Typical fluorescence vs time profile of trypsin (---•---) Kentucky blue grass diffusate (------) and PBS (·····◆·····) after incubation with Nα-benzoyl-L-Arg 7-amido-4-methylcoumarin for 1 min then fluorescence measured every min for 70 min. Fluorescence of Bermuda grass reached a similar level and rye grass was ~ 2 × > Kentucky blue grass diffusate after 60 min. B. Pollen diffusates of Kentucky blue grass (■), rye grass (■) and Bermuda grass (■) (± inhibitors) were incubated with NBAMC for 1 min, then fluorescence measured after ~ 60 min. Maximum fluorescence was generally reached at ~ 60 min and values obtained after addition of inhibitors are expressed as relative reduction in activity compared to diffusates alone (100%).

that the activity present in Kentucky blue grass was almost entirely specific for the basic amino acids [23], the results from the current experiments indicate otherwise. We believe this might be a consequence of the relative rates of lysis of different peptide substrates being possibly influenced by the assay conditions or substrate. The previous study employed an assay in which the initial pH of the reaction mixture was markedly alkaline and activity was revealed by an indicator dye sensitive to the progressive fall in pH.

Trypsin-like peptidase activity in pollen diffusates is well established. Mesquite (*Prosopis velutina*) and ragweed (*Ambrosia artemisiifolia*) pollens contain peptidases with trypsin-like and chymotrypsin-like specificities [18,19]. However, the function of these pollen enzymes remains largely unknown.

In addition to peptidases, pollen diffusates may contain novel endoproteases with sequence identity to the group 1 allergens [20]. Therefore, we assayed the 3 pollen diffusates for protease activity using gelatin zymography.

SDS/PAGE and Zymography

All pollen diffusates analysed contained relatively high protein concentrations ($\sim$ 2–5 mg/ml) and SDS/PAGE analysis showed intensely-stained bands of M_r ranging from <10,000 to $\sim$ 100,000 (Fig 2). Previous reports have demonstrated numerous bands within each pollen diffusate, corresponding to allergenic proteins [28]. Diffusates of Kentucky blue (Fig 3, lanes 2–6), rye (Fig 3, lanes 7–11) and Bermuda grasses (Fig 3, lanes 12–16) were tested for proteolytic activity using gelatin and casein zymography. Gelatin zymograms generally revealed more intense activity and were used for most experiments. All zymograms contained several intense negatively-stained bands at $M_r \sim$ 95,000 (Fig 3, lanes 2, 7 and 12) possibly corresponding to a common protease. A proteolytic band at $M_r \sim$ 65,000 was evident in rye grass diffusate and Bermuda grass diffusate also contained an additional proteolytic band at $M_r \sim$ 35,000 (Fig 3, lane 12). All proteolysis was fully inhibited by the complete protease inhibitor cocktail (Fig 3, lanes 4, 9 and 14), partially inhibited by AEBSF (Fig 3, lanes 5, 10 and 15) whereas little inhibition was observed with iodoacetamide (Fig 3, lanes 6, 11 and 16). These results suggest that these components have serine protease activity.

A monoclonal antibody specific to epitopes on the Phl p 1 allergen from timothy grass (*Phleum pratense*) was tested using Western blotting. One intense band $M_r \sim$ 35,000, which corresponded to the predicted M_r of the major group 1 allergen, was readily detected in Kentucky blue and Bermuda grass diffusates. The immunoreactivity indicates common epitopes and suggests common functions of these allergens in pollen. The *Phleum pratense* group 1 allergen (Phl p 1) has 70%, 90% and 93% identity to homologous group 1 allergens derived from *Poa pratensis* (Poa p 1), *Lolium perenne* (Lol p 1) and *Cynodon dactylon* (Cyn d 1) respectively [29]. Group 1 allergens are pollen glycoproteins of predicted $M_r \sim$ 30,000, that are released after hydration. They are a subset of the b-expansin family of genes in plants [30]. Sequence similarities to expansins, and functional studies, showed that the group I allergen from maize pollen (Zea m 1) exhibits expansin activity specifically towards grass cell walls [30]. Expansins are
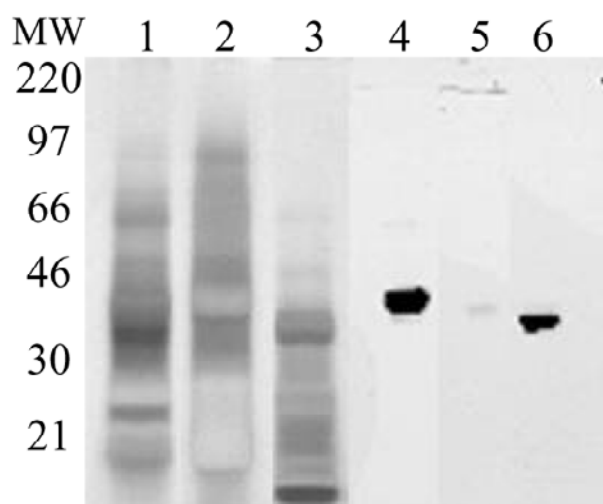

Figure 2
SDS/PAGE and Western blot analysis of pollen diffusates. Approximately 10 μg of diffusate was loaded in each lane of a 12.5% SDS/PAGE and silver-stained (lanes 1–3). Lane 1, Kentucky blue grass (*Poa pratensis*); Lane 2, rye grass (*Lolium perenne*) and Lane 3, Bermuda grass (*Cynodon dactylon*): Lane 4, Kentucky blue grass; Lane 5, rye grass and Lane 6, Bermuda grass after Western blotting with a monoclonal antibody (IG 12) to allergen Phl p 1. MW markers are shown.

defined by their characteristic function and sequence similarities. Grobe et al [20] have proposed that timothy grass group 1 allergen (Phl p 1) possesses proteolytic activity, demonstrable after pre-incubation under reducing conditions. The authors suggested that the amino acid sequence of Phl p 1 contained a cysteine protease motif similar to that found in papain. However, there are conflicting reports about whether the observed proteolytic activity is due to Phl p 1 itself or a contaminating protease [20,21,31,32]. The M_r of the proteolytic band at $\sim$ 35,000 observed in Bermuda grass diffusate was close to the observed M_r of the Phl p 1 immuno-reactive band detected in the Western blot (Figs 2 and 3). Because of their high sequence identity, it is possible that the group 1 allergen of Bermuda grass (Cyn d 1) shares epitopes with Phl p 1 and may also possess proteolytic activity. SDS/PAGE and zymograms were repeated, but it was not possible to accurately align silver-stained gels, zymograms and Western blots to determine if Cyn d 1 was the protein responsible for the proteolytic activity. Therefore, we undertook additional studies using two-dimensional gel electrophoresis and mass spectrometry as described below.
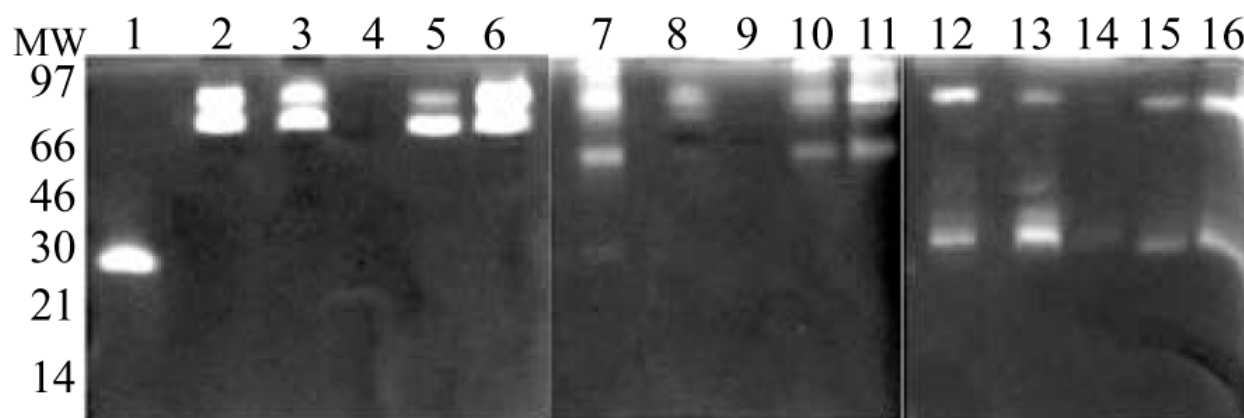
Figure 3
Gelatin zymography of pollen diffusates and sensitivity to inhibitors. Approximately 10 μg of Kentucky blue grass (lanes 2–6) rye grass (lanes 7–11) and Bermuda grass (lanes 12–16) diffusates were separated on 12.5% SDS/PAGE. Trypsin (~500 ng) was loaded in lane 1. Diffusates (lanes 2, 7 and 12) contain several high MW bands (~95,000) with proteolytic activity and Bermuda grass diffusate also contains intense proteolytic activity at ~35,000 (lane 12). PMSF partially inhibited some of the activity in rye grass (lane 8) but not Kentucky or Bermuda (lanes 3 and 13); complete protease inhibitors (lanes 4, 9 and 14) totally blocked activity; and AEBSF (lanes 5, 10 and 15) was a moderately effective inhibitor. Activity was unaffected by iodoacetamide (lanes 6, 11 and 16).

Proteomic analysis of pollen diffusates
A proteomic approach, enabling rapid identification and analysis of proteins, was employed to confirm the identities of the bands surrounding the proteolytic activity at M_r ~ 35,000. The identities of other intensely staining bands were also examined to verify that mass spectrometry was suitable for analysis of pollen proteins. Bands were excised from diffusates of Kentucky blue grass, rye grass and Bermuda grass that had been separated by SDS/PAGE and stained using CBB (Fig 4a), and were digested with trypsin. Peptides were analysed by MALDI reflectron TOF peptide mass fingerprinting and micro-LC/ESI low energy CID MS/MS.

Peptide mass fingerprinting of pollen difusates
Figures 4b and 4c show MALDI reflectron TOF spectra of tryptic peptides of band 4 from rye grass and band 12 from Bermuda grass diffusates. Intense ions corresponding to protonated tryptic peptides of proteins present in each band were readily detected, with mass errors of < ~ 100 ppm. Database searches using peptide masses from each spectrum indicated that the most likely protein in band 4 was the allergen Lol p 1 (rye grass) and that in band 12 was the allergen Cyn d 1 (Bermuda grass). Five additional proteins corresponding to known pollen allergens were readily identified by peptide mass fingerprinting from the 16 excised bands analysed (data not shown). These corresponded to some of the most

intensely staining bands observed in the gel. While MALDI peptide mass finger printing is a sensitive, quick and specific approach, bands containing several proteins are more difficult to identify because the peptide masses may correspond to the theoretical digest of an unrelated protein leading to false positives after database searching [33]. To circumvent this problem, diffusates were reanalysed using two-dimensional electrophoresis, followed by extraction of the tryptic digests of the protein spots from the gels, analysis of the sequence tags derived from LC separation and low energy CID MS/MS, and database searching. This approach allows easy identification of peptide mixtures [27,34].
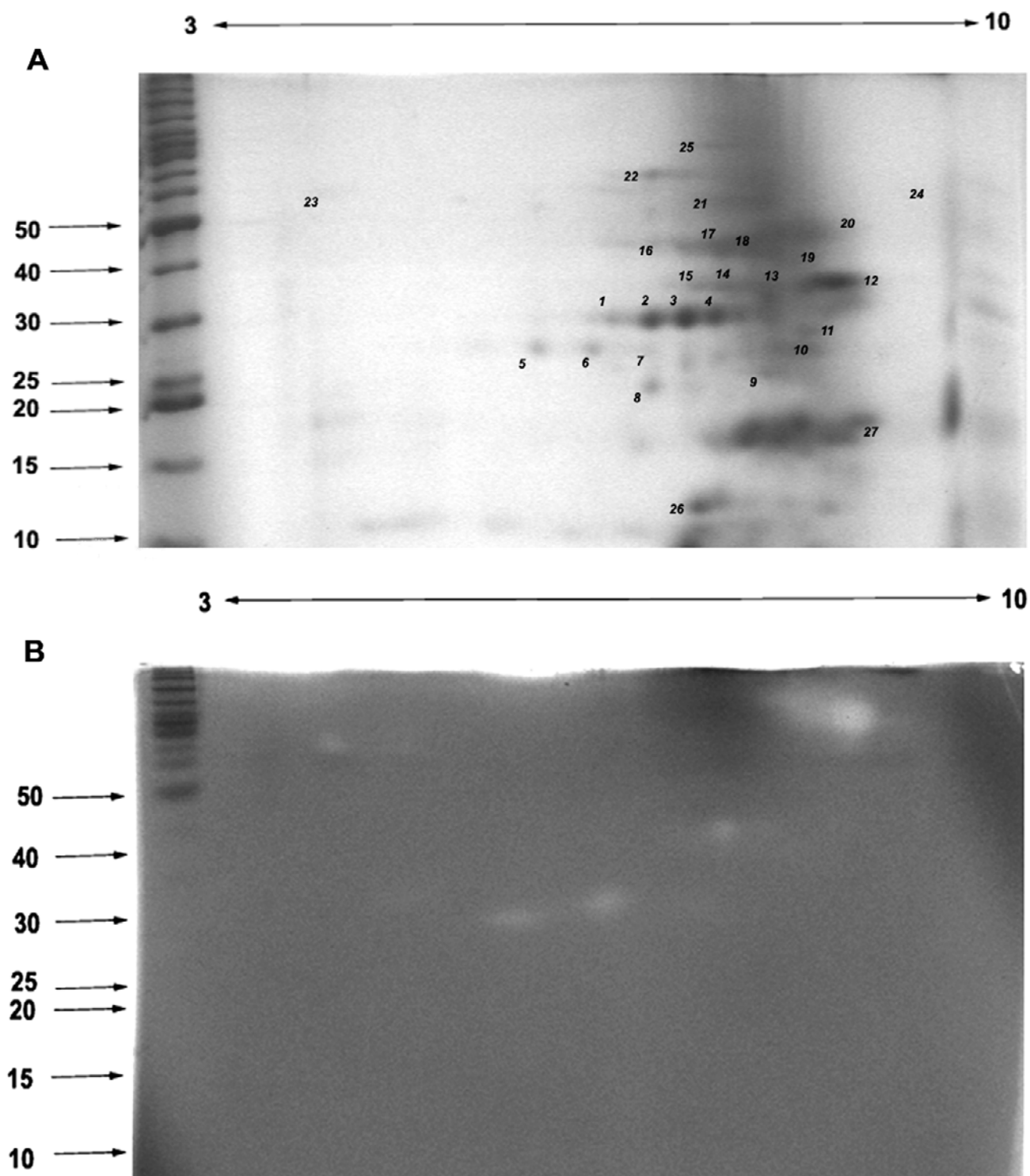
Two-Dimensional SDS/PAGE and Zymography
Two-dimensional electrophoretic separation of the concentrated diffusate yielded a number of protein spots at Mr 30,000–35,000 Da. Isoelectric focusing/gelatin zymography of the diffusate yielded diffuse proteolytically active streaks around the same region (Fig 5). Relatively weaker proteolytic activity in the two-dimensional zymograms might have been a consequence of extreme conditions of denaturation and subsequent renaturation.

*Micro-LC/MS of pollen diffusates*
The proteolytic digests of the protein spots from the isoelectric focusing SDS/PAGE of Bermuda grass diffusate were separated by micro C18 RP HPLC followed by auto-
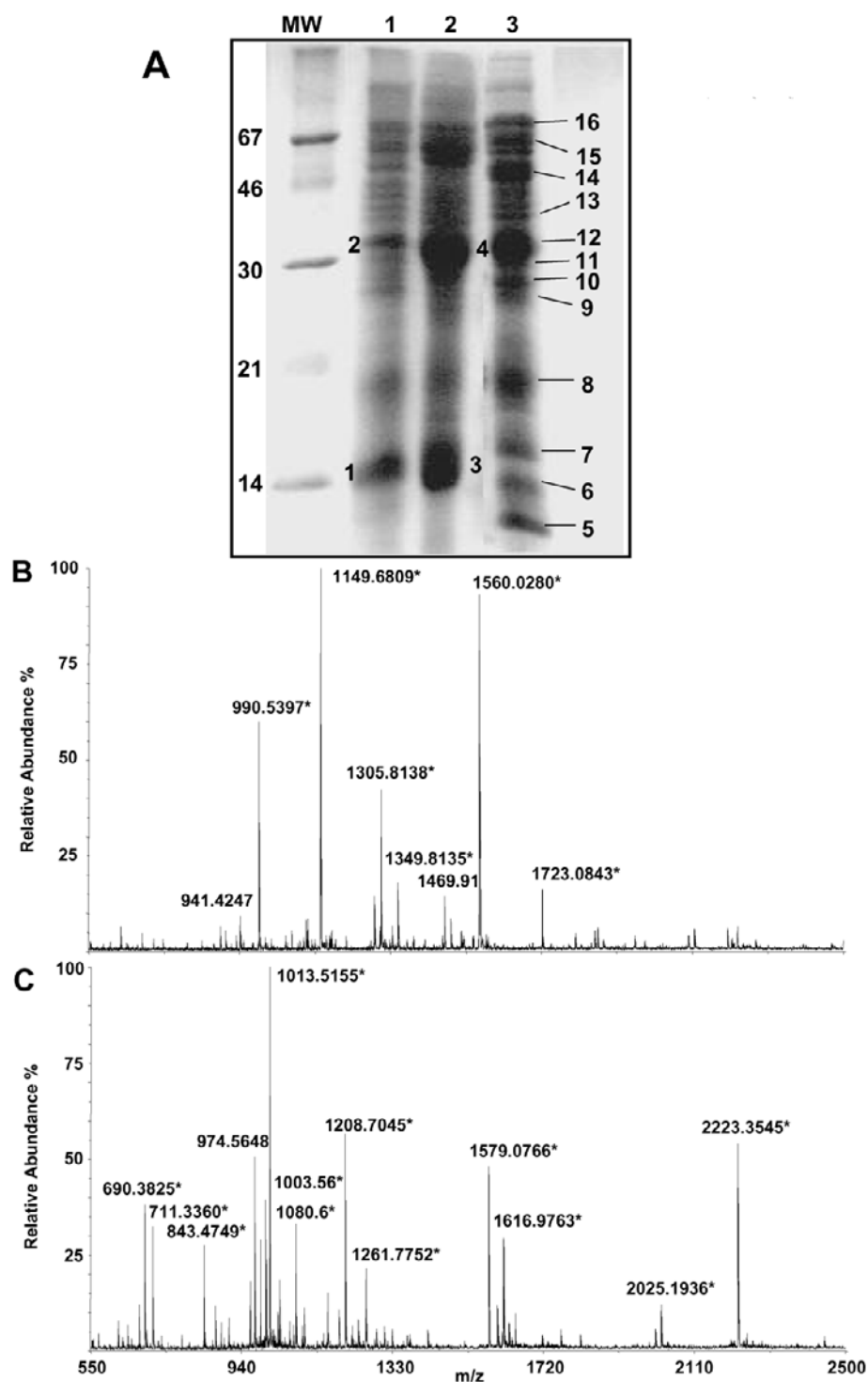
Figure 4
SDS/PAGE and MADLI reflectron TOF of tryptic peptides derived from band 4 (*Lolium perenne*) and band 12 (*Cynodon dactylon*). A. Pollen diffusates (~ 20 μg: lane 1 Kentucky blue grass, lane 2 rye grass and lane 3 Bermuda grass) were separated using SDS/PAGE, stained with CBB and labelled bands excised, destained and digested with trypsin. B. Peptides from band 4 (rye grass) were analysed using MALDI reflectron TOF; digest peptides with masses corresponding to allergen Lol p 1 are labelled (*). C. Peptides from band 12 (Bermuda grass) were analysed using MALDI reflectron TOF, digest peptides with masses corresponding to allergen Cyn d 1 are labelled (*).

Figure 5
Two-dimensional SDS/PAGE and zymography of Bermuda grass diffusate. A. Two-dimensional SDS/PAGE stained with CBB; spots cut and digested with trypsin for micro-LC/MS-MS are labelled. B. Corresponding gelatin zymogram; negatively-stained spots of lower M_r correspond to spots identified as Cyn d 1.
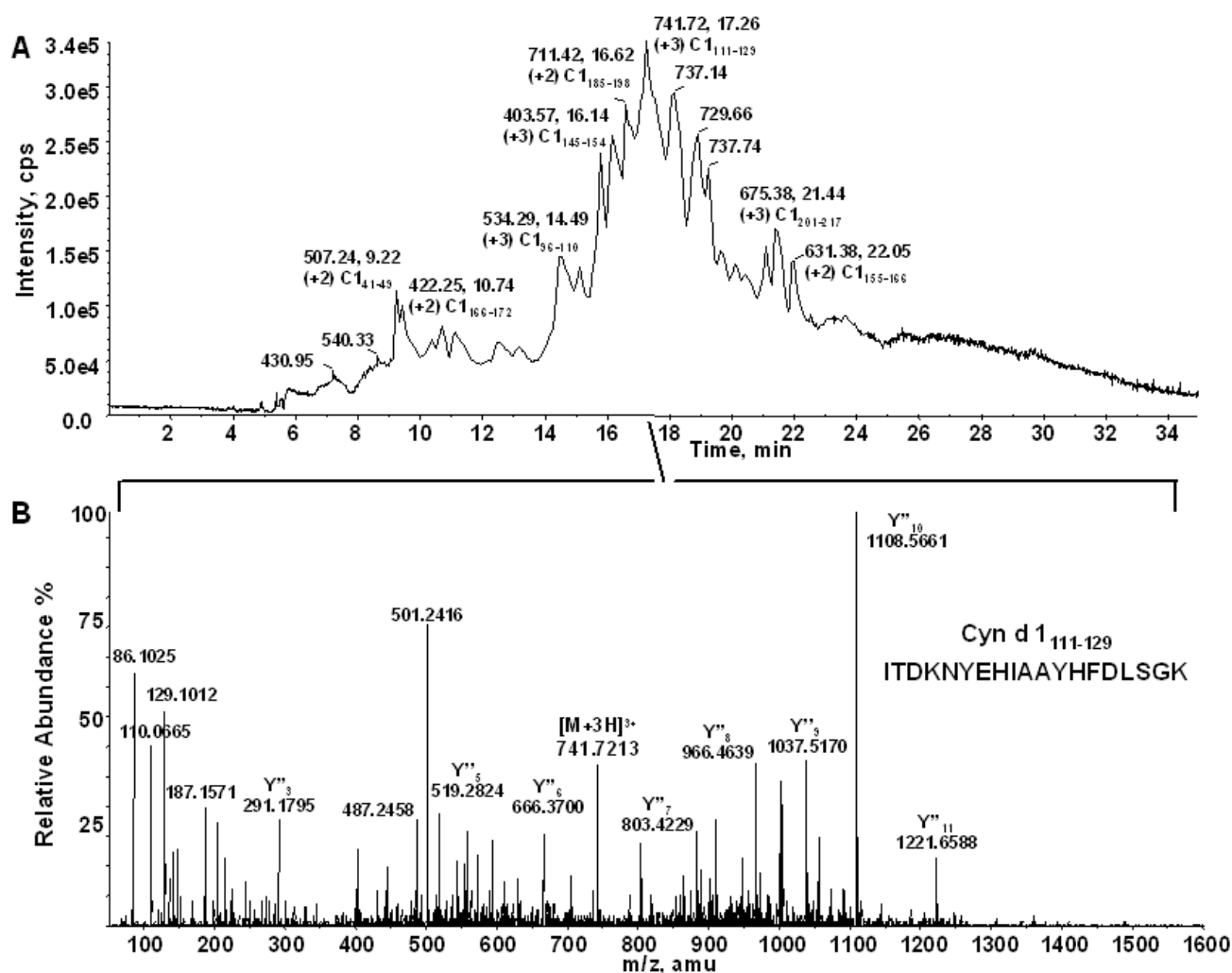
Figure 6
Micro C18 RP-HPLC electrospray MS/MS analysis of tryptic peptides derived from spot 4 (*Cynodon dactylon*).
Peptides from tryptic digestion of spot 4 (Fig 5a) were separated using micro C18 RP-HPLC and the eluate analysed by ESI-MS/
MS. A. Base peak mass chromatogram, each peak is labelled with the retention time, mass, charge state and identity deter-
mined by low energy CID tandem MS of the multiply-charged precursor ions. B. MS/MS spectrum of a triple charged precursor
ion m/z 741.7 corresponding to the sequence ITDKNYEHIAAYHFDLSGK from allergen Cyn d 1 was identified by database
searches (Mascot).

mated data-dependent low energy CID MS/MS analysis of
the most intense multiply charged-peptide ion identified
in the eluting peptides [27]. Peptide sequence tags from
the entire chromatogram were used for database searches.
The base peak mass chromatogram of tryptic peptides
from spot 4 is shown in Figure 6a. Typical MS/MS spectra
from multiply-charged peptide ions are shown in Fig 6b.
After database searches of all spectra, spot 4 was identified
as allergen Cyn d 1. Intense ions corresponding to

sequential cleavage of the $[M+3H]^{3+}$ ion of Cyn d $1_{111-129}$
(isoform 1) were readily identified and an almost com-
plete series of y-type fragment ions was present (Fig 6b).
The identification of multiple peptide sequence tags from
the same protein allowed identification with a high
probability.

For each of the excised and digested spots, micro-LC/MS
followed by semi-automated database searches were per-

formed. Table 1 summarises proteins identified. Searches performed on the amino sequence tags determined after micro-LC separation and MS/MS permitted identification of peptides from protein mixtures, but because the current plant protein sequence databases are incomplete, in some cases the proteins identified were from other plant species, indicating that the derived sequence tags may be common to two or more species.

The spots that separated at M_r 30,000–35,000 and co-migrated with the observed proteolytic activity were identified by micro-LC ESI-MS/MS as the group 1 allergen Cyn d 1 from Bermuda grass.

Conclusion

SDS/PAGE and mass spectrometry proved quite useful in rapidly determining the identities of pollen proteins after digestion with trypsin. In the future, this approach will allow identification and differentiation of allergen isoforms and of post-translational modifications. Our data strongly suggest that, analogous to house dust mite and other allergens, pollen allergens may also be proteases. The major proteolytically-active band in Bermuda grass, which localised at $M_r \sim 35,000$ in one-dimensional electrophoresis, was the group 1 allergen Cyn d 1. Similarly, the major proteolytically active spots at approximately the same Mr in two-dimensional electrophoresis were also identified as Cyn d 1, thus strongly suggesting that this allergen has intrinsic proteolytic activity. While confirmation of this result by examination of recombinant Cyn d 1 could be useful, the well documented contamination of such preparations by proteases from the expression system (e.g. yeast) [32] may limit the value of such additional experiments. High purity isolates of native Cyn d 1 may be more reliable for such assessment.

Abbreviations

AEBSF: 4-(2-aminoethyl)benzenesulfonylfluoride; CBB: Coomassie brilliant blue; CID: collision-induced dissociation; ESI: electrospray ionisation; MALDI: matrix-assisted laser desorption ionisation; MS: mass spectrometry; NBAMC: Nα-benzoyl-L-Arg 7-amido-4-methylcoumarin; PMSF: phenylmethylsulfonyl fluoride; TLCK: Tosyl Lys chloromethyl ketone; TPCK: Tosyl Phe chloromethane ketone; SDS/PAGE: sodium dodecyl sulphate/polyacrylamide gel electrophoresis; TOF: time of flight.

Authors' contributions

MJR supervised the experimental work, performed characterisation by mass spectrometry and contributed to drafting the manuscript. RGS carried out assays for enzymatic activity, performed gel electrophoresis and contributed to drafting the manuscript. CLG and RKK helped with the design and interpretation of studies and with manuscript

preparation. All authors read and approved the final manuscript.

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