

Substrate preference profiles of proteases released by allergenic pollens

F. WIDMER, P. J. HAYES, R. G. WHITTAKER and R. K. KUMAR*

CSIRO Molecular Science, North Ryde and *School of Pathology, University of New South Wales, Sydney, Australia

Summary

Background Pollens are important triggers for allergic asthma and seasonal rhinitis. We have recently reported that proteases released by major allergenic pollens can injure airway epithelial cells *in vitro*. Disruption of epithelial integrity by proteases released following deposition of pollens on mucosal surfaces could promote sensitization and induce inflammation.

Objective To compare protease activities released by allergenic pollens of various genera.

Methods We used a rapid microassay which quantifies cleavage of dipeptide ester substrates to characterize the substrate preference profiles of serine proteases in diffusates of the pollens of perennial ryegrass (*Lolium perenne*), Kentucky blue grass (*Poa pratensis*), Bermuda grass (*Cynodon dactylon*), Western ragweed (*Ambrosia* spp.), white birch (*Betula* spp.) and Sydney golden wattle (*Acacia longifolia*).

Results Comparison of the profiles revealed notable differences as well as similarities between serine protease activities released by these pollens. Diffusates of Kentucky blue grass pollen exhibited very high substrate preference for arginine and lysine. For other pollens, cleavage of the cysteine substrate was usually the most rapid and was associated with marked preference for leucine and methionine. There was considerable variation between these pollens in the rates of cleavage of the histidine substrate. In addition, we observed high rates of cleavage of arginine and lysine substrates by *Acacia* pollen diffusate.

Conclusion At least two dominant patterns of substrate preference are identifiable in the mixtures of proteases released by hydrated pollens. Purification of the proteases responsible for these patterns of activity will facilitate investigation of their role in airway epithelial injury and allergic disease.

Keywords: airway epithelium, allergic rhinitis, asthma, pollens, proteases

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Introduction

In the majority of patients who develop asthma or seasonal rhinitis/conjunctivitis, the basis of the mucosal inflammatory response appears to be IgE-mediated allergic sensitization to airborne allergens [1,2]. The reasons for the very high incidence of allergic asthma and rhinitis in some parts of the world, including Australia, remain incompletely defined. Environmental factors are likely to be important, a notion reinforced by evidence that the prevalence of asthma and rhinitis in immigrants to Australia increases

significantly with the length of stay [3]. Experimental studies suggest that both genetic and environmental factors influence the induction of an IgE response to an inhaled antigen [4]. Thus in susceptible individuals, an IgE response may be stimulated if the integrity of the airway epithelial barrier is breached at the time of initial exposure, for example by concurrent infection or exposure to environmental pollutants. This is presumably a result of facilitated access of allergenic protein components to the subepithelial antigen-presenting dendritic cells [5].

In this context, it is therefore noteworthy that several important allergens of the house dust mite [6,7] as well as cockroach allergens [8] have intrinsic protease activity.

Correspondence: R. K. Kumar, School of Pathology, University of New South Wales, Sydney, Australia 2052.

Exposure to proteases *in vitro* causes epithelial detachment and increases the permeability of epithelial sheets and cultured monolayers of airway epithelium [9,10]. Epithelial denudation is not necessarily involved in increasing epithelial permeability, as these enzymes may cause disruption of tight junction-associated proteins [10]. *In vivo*, injury to airway epithelial cells by this mechanism could promote sensitization to antigens that possess intrinsic protease activity [7,9].

Airborne allergens derived from plant pollens are important triggers for asthma and rhinitis. Information about whether allergenic proteins of pollens might function as proteases is lacking [11] even though the presence in pollens of a variety of enzymes, including proteases, has long been recognized [12]. We hypothesized that liberation of such enzymes upon inhalation of pollens could injure the respiratory epithelium. Consistent with this hypothesis, we have recently demonstrated that proteases released by pollens of perennial ryegrass (*Lolium perenne*), Kentucky bluegrass (*Poa pratensis*), she-oak (*Casuarina distyla*) and Sydney golden wattle (*Acacia longifolia*) are able to cause concentration-dependent detachment of airway epithelial cells in culture [13]. Moreover, enzymatic activity was not blocked by major endogenous antiproteases, including α_1 -proteinase inhibitor and secretory leucocyte protease inhibitor. Disruption of the epithelial barrier by proteases in pollens might thus promote sensitization to these proteins or to simultaneously released pollen antigens with no intrinsic enzymatic activity [13].

Characterization of the protease activities in pollens can be achieved by purification of individual enzymes and examination of their substrate specificity and susceptibility to inhibitors. Using such an approach, Travis and coworkers have described the properties of several proteases from ragweed and mesquite pollens [14–16]. However, because of the large number of proteases that may be released by pollens of individual species, this is a labour-intensive strategy. In the present study, we have used a rapid microplate assay [17] to examine the substrate specificity profiles of unfractionated serine proteases released by several potentially allergenic pollens.

Materials and methods

Preparation of pollen diffusates

Pollens of ryegrass (*L. perenne*), Kentucky blue grass (*P. pratensis*), Bermuda grass (*C. dactylon*), Western ragweed (*Ambrosia* spp.), white birch (*Betula* spp.) and Sydney golden wattle (*A. longifolia*) were purchased from Bayer Australia, Sydney. Diffusates of the pollens were prepared under physiological conditions by thoroughly mixing 200 mg of dry pollen with 2 mL of normal saline and incubating without agitation at 37°C for 30 min. The

pollen was then centrifuged at 16 000 g for 8 min and washed three times in 2-mL volumes of saline. Supernatants were pooled and reconcentrated to 1 mL for assessment of enzymatic activity.

Assessment of protease activity and substrate preference

Serine protease activity in pollen diffusates was characterized using a modified version of a rapid microplate assay which quantifies cleavage of dipeptide ester substrates [17]. Because all serine proteases are able to cleave both peptide bonds and peptide ester bonds, and because enzymes other than proteases do not cleave these bonds effectively, this provides a useful alternative to conventional assays. The substrates take the form Z-Ala-X-OMe, where X may be any of the 20 common amino acids. Release of unbuffered protons from cleaved ester substrates is quantified colourimetrically using phenol red as an indicator dye. Assay conditions were optimized to cater for potential interference with the colour reaction by pollen pigments and the starting pH of all reaction mixtures was adjusted to 8.3 in the presence of 25% dimethyl sulphoxide. Absorbance measurements at 560 nm were taken at 30-s intervals for 30 min and the initial reaction velocity, which is proportional to enzymatic activity, was determined. Two completely independent assays were performed for each pollen. To simplify comparison between pollens, data were expressed as a percentage of the reaction rate for the most rapidly cleaved substrate in each assay. Results are presented as mean $\pm$ SEM.

Results

The rates of substrate cleavage by the various pollen diffusates were similar in absolute terms, ranging from 0.01 to 0.02 OD units/min for the most rapidly cleaved substrate for all pollens. However, there were noteworthy differences in substrate preference profiles.

As shown in Fig. 1, serine protease activity in the diffusate of *P. pratensis* pollen exhibited a marked preference for arginine and lysine in the P₁ position. There was relatively little cleavage of any other substrate by this diffusate.

In contrast, the profiles of *Lolium*, *Cynodon* and *Ambrosia* pollen diffusates revealed low rates of cleavage of arginine and lysine substrates, but were remarkably similar in terms of their preference for cysteine, leucine and methionine substrates. The profile of activity released by *Betula* pollen was comparable, although relative preference for leucine and methionine was lower.

These four pollen diffusates also cleaved the glutamine substrate to an equivalent extent, as well as cleaving alanine and phenylalanine substrates with relative activities varying between 20% and 60% for alanine and 15–40% for

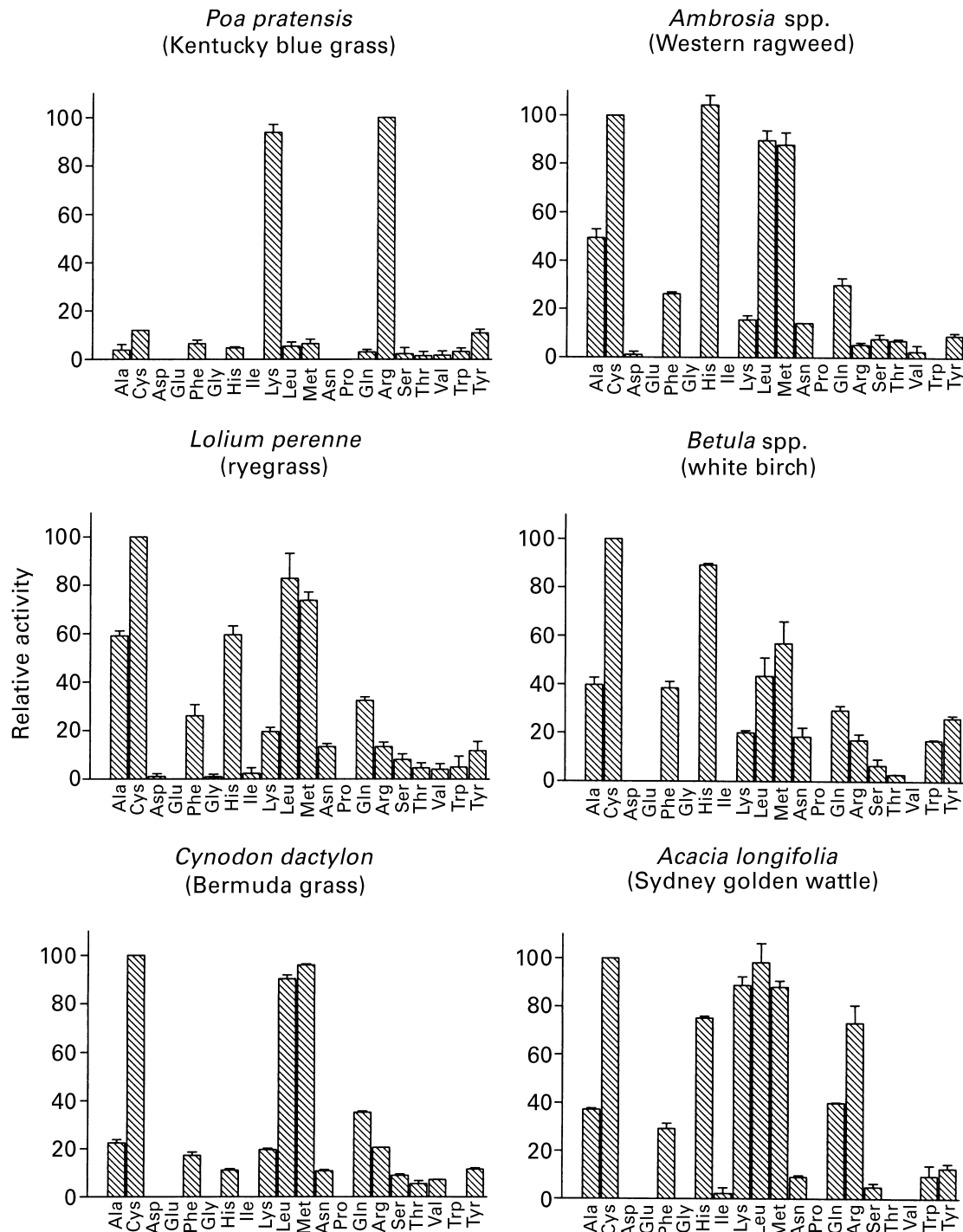


Fig. 1. Cleavage of dipeptide ester substrates by diffusates of various pollens. The amino acid in the P₁ position of each substrate is shown. Relative activity is normalized to arginine for *Poa pratensis* and to cysteine for other pollens. Results are presented as mean $\pm$ SEM of two independent assays.

phenylalanine. However, there were striking differences between the pollens with respect to activity for the histidine substrate, which ranged from 10% of maximum for *Cynodon* to 100% of maximum for *Ambrosia*.

The substrate preference profile of *Acacia* pollen diffusate appeared to include features of both patterns described above. Thus in addition to rapid cleavage of cysteine, leucine and methionine, this pollen also released high

	<i>Poa</i>	<i>Lolium</i>	<i>Cynodon</i>	<i>Ambrosia</i>	<i>Betula</i>	<i>Acacia</i>
Alanine	+-	+++	+	++	++	++
Cysteine	+	++++	++++	++++	++++	++++
Histidine	+-	+++	+	++++	++++	+++
Lysine	++++	+	+	+	+	++++
Leucine	+	+++	++++	++++	++	++++
Methionine	+	+++	++++	++++	+++	++++
Arginine	++++	+	++	+	+	+++

Table 1. Relative cleavage of selected substrates by pollen diffusates

levels of activity for arginine and lysine substrates, together with activity for the histidine substrate.

Key similarities and differences between the relative activities of the pollen diffusates for selected substrates are summarized in Table 1.

Discussion

Pollens release a variety of enzymes upon hydration, including proteases. Our previous studies have established that pollen proteases may be biologically important, as they are able to cause epithelial damage and may not be inhibited by endogenous antiproteases [13]. Indeed, some of these enzymes may be able to cleave antiproteases such as human α_1 -proteinase inhibitor [15]. Further characterization of proteases released by pollens is therefore of considerable interest.

However, all pollen diffusates contain a mixture of enzymes including multiple proteinases and/or peptidases, making it difficult to determine which of a number of activities is most appropriate for further study. Purification of individual proteases released by pollens is labour-intensive. The experiments reported here demonstrate the potential of screening for substrate preference using a novel microplate assay. The technique relies on the esterase activity of proteases which, as has been demonstrated for the bacterial enzyme thermitase, may not be proportional to the peptidase activity [18]. Nevertheless, this assay is useful for identification of dominant enzymatic activities.

Our data are consistent with a single enzyme being responsible for most of the serine protease activity in diffusates of *Poa* pollen, although this remains to be formally established. A pattern of shared substrate preference for cysteine, leucine and methionine was demonstrated for pollens of *Lolium*, *Cynodon*, *Ambrosia* and *Betula* species, perhaps indicating the presence of one or more common enzymes. The profile of activity in diffusates of *Acacia* pollen differed from those pollens principally with respect to increased cleavage of arginine and lysine substrates, which could be interpreted as indicating the

additional presence of an enzyme similar to that in *Poa* diffusates. We recognize that the diffusates might also contain cysteine protease activity. The microplate assay has been used successfully to characterize the substrate preference of such enzymes (R. G. Whittaker and P. J. Hayes, unpublished results) and assessment of cysteine proteases in pollen diffusates is ongoing.

The putative dominant serine protease in diffusates of *Poa* pollen exhibits a marked substrate preference for arginine and lysine in the P₁ position, which is very similar to the activity profiles of trypsin and thrombin that we have reported previously [17]. Furthermore, it appears to be similar to that described for a peptidase in mesquite pollen [14], as well as resembling a trypsin-like peptidase with substrate preference for arginine but not lysine that has recently been purified from ragweed pollen [16]. However, our data are based entirely upon dipeptide substrates synthesized with alanine in the P₂ position, which may preferentially enhance [17] or obscure [15] detection of the cleavage of some substrates.

The substrate preference profiles of activity in the other five pollen diffusates that we studied were more complex and thus more likely to reflect the presence of multiple enzymes. Nevertheless, purified bacterial subtilisin has a similar complex profile [17] with marked preference for cysteine, leucine and methionine substrates, as well as exhibiting activity for glutamine and phenylalanine substrates. Therefore the presence of a single dominant enzymatic activity common to all of these pollens cannot be excluded. Of interest is the apparent presence in some of these pollens of an additional histidine-specific protease.

When pollen is deposited on a mucosal surface, protease enzymes are likely to be rapidly released, because more than two-thirds of the total extractable solutes are liberated from the outer layers of the pollen grain within minutes of hydration [19]. For this reason, we examined serine protease activity in pollen diffusates, deliberately employing an approach which recovers soluble components released after hydration without mechanical disruption. *In vivo*, this rapid release of solutes may generate very high local

concentrations in the microenvironment of the pollen grain, of the order of 50 mg/mL [20]. The liberated protease enzymes could disrupt epithelial tight junctions, facilitating transepithelial transport of proteins, which might promote sensitization as a result of increased access of allergenic proteins to the subepithelial antigen-presenting dendritic cells [5,7]. In addition, tissue damage by proteases could directly induce an inflammatory response. These enzymes might also promote inflammation as a consequence of proteolytic activation of inflammatory mediators [21].

Because of the size of pollen grains, the primary site of epithelial injury by proteases released by inhaled pollens would probably be the mucosa of the upper respiratory tract. However, rupture of individual pollen grains upon hydration releases hundreds of smaller starch granules which are potentially respirable [22]. Whether protease activity is associated with or subsequently released by these granules is unknown.

Further studies of the protease(s) responsible for the distinct patterns of substrate preference we have observed will require isolation of the relevant enzyme(s). This will permit assessment of their biological importance, including characterization of the class and active site of the proteases using appropriate inhibitors, examination of their ability to cleave relevant peptide and protein substrates, as well as comparison of amino acid sequences with those of known pollen allergens. It would also be of interest to directly evaluate the ability of purified proteases to promote sensitization via the respiratory tract in a bioassay, possibly using intranasal exposure as has been employed for other enzymes [23].

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