

Pollen proteases compromise the airway epithelial barrier through degradation of transmembrane adhesion proteins and lung bioactive peptides

R. Vinhas¹, L. Cortes¹, I. Cardoso¹, V. M. Mendes¹, B. Manadas¹, A. Todo-Bom², E. Pires^{1,3} & P. Veríssimo^{1,3}

¹Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal; ²Immunology Service, Coimbra University Hospital, Coimbra, Portugal; ³Department of Life Sciences, University of Coimbra, Coimbra, Portugal

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Correspondence

Paula Veríssimo, Ph.D., Center for Neurosciences and Cell Biology of Coimbra, University of Coimbra, 3004-517 Coimbra, Portugal.

Tel.: +351 239 104 473

Fax: +351 239 822 776

E-mail: paulav@imagem.ibili.uc.pt

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Abstract

Background: Allergic disorders, such as seasonal rhinitis and asthma, are increasing causes of morbidity worldwide and often result from exposure to airborne pollen. Pollen allergy has a remarkable clinical impact all over Europe. In fact, epidemiological longitudinal studies confirm that pollen species usually considered with low allergenic potential became more recently responsible for intense allergic reactions. In this study, we aimed to characterize major pollen proteolytic activity and evaluate its contribution to the immunologic and inflammatory response to airborne allergens.

Methods: Proteolytic activity in four pollen diffusates with distinct allergenicity, *Olea europaea*, *Dactylis glomerata*, *Cupressus sempervirens* and *Pinus sylvestris*, was evaluated through several enzymatic assays. The action of pollen proteases on the paracellular integrity of Calu-3, grown at the air–liquid interphase, was evaluated through a transepithelial permeability assay. Immunoblot and immunofluorescence experiments were performed to analyse the disruption of intercellular complexes. Degradation of bioactive peptides by pollen crude extracts was assessed by mass spectrometry.

Results: All pollen diffusates were shown to have high molecular weight proteases with serine and/or aminopeptidase activity. These proteases increased Calu-3 transepithelial permeability through disruption of transmembrane adhesion proteins: occludin, claudin-1 and E-cadherin. Moreover, they were able to degrade airway bioactive peptides and were not blocked by endogenous protease inhibitors.

Conclusion: Pollen grains with distinct allergenic abilities release proteases that might be involved in the sensitization to a range of airborne allergens by facilitating allergen delivery across the epithelium and also contribute directly to the inflammation characteristic of allergic diseases.

Pollen is an important trigger for allergic disorders – a major health issue that affects about 40% of the European population (1). Asthmatic patients allergic to pollen develop a chronic inflammation of the airways, bronchial obstruction and hyperresponsiveness to several triggers. However, the

mechanisms that set off an allergic reaction and an inflammatory response after pollen exposure are not fully understood. Once inhaled, pollen grains become hydrated and release a wide variety of allergens and other proteins, for instance, proteases, NADPH oxidases, and PGE 2- and LTB 4-like substances that may act as inflammatory mediators (2–5).

A number of studies describe several pollen proteases that might contribute to the pathogenesis of respiratory allergies. Ragweed, mesquite and *Parietaria judaica* pollens have been shown to release different proteolytic enzymes involved in the inactivation of lung regulatory neuropeptides, such as

Abbreviations

AMC, 7-amino-4-methylcoumarin; DC, dendritic cell; IPG, immobilized pH gradient; PAR, protease-activated receptor; SLPI, secretory leukoprotease inhibitor; VIP, vasoactive intestinal peptide; ZO-1, zonula occludens-1.

substance P and vasoactive intestinal peptide (VIP) (6–10). *In vivo*, inactivation of bioactive peptides may alter the balance between contractile and relaxant effects in the respiratory airways.

During the sensitization process, inhaled allergens should cross the epithelial barrier to be processed by dendritic cells (DCs) located in subepithelial regions. Then, activated DCs present allergen peptides to naïve T lymphocytes that elicit specific IgE production. Allergens are sampled by DCs via interepithelial pseudopods or by gaining access to DCs through cleavage of proteins from junctional complexes between epithelial cells (11). In fact, a 98-kDa aminopeptidase purified from *P. judaica* causes detachment of human alveolar epithelial cells (A549) by degrading intercellular adhesion proteins from tight junctions and adherens junctions (10). Furthermore, diffusates from four highly allergenic pollens, *Ambrosia trifida*, *Poa pratensis*, *Betula pendula* and *Lilium longifolium*, cause loss of immunofluorescence labelling of tight junctions proteins on epithelial cell lines. This effect is blocked by serine and cysteine protease inhibitors (12).

This study is focused on four pollen types with different allergenic capacities that are widespread over the Mediterranean area: *Olea europaea* (Olive tree – high allergenicity), *Dactylis glomerata* (Orchard grass – high allergenicity), *Cupressus sempervirens* (Italian cypress – moderate allergenicity) and *Pinus sylvestris* (Scots pine – low allergenicity) (1, 13, 14). Another interesting feature of these species is related to their partly overlapping pollination seasons in southern Europe. Pollination by the olive tree and grasses lasts from April to July and is recognized as being one of the most important causes of seasonal respiratory allergies in the Mediterranean area (1, 13). Cypress pollination occurs mainly from December to March and is characterized by very high concentrations in the Mediterranean coastal, where it often induces rhinoconjunctivitis (1, 13). Pine pollen has long been considered a nonallergenic pollen; however, it is responsible for very high pollen counts throughout the year, especially from March to May (14, 15). Thus, we decided to characterize the major proteolytic activity found in these pollen diffusates, identify potential targets once pollen proteases reach the respiratory system and investigate how they affect the epithelial barrier function and bioactive peptides homeostasis.

Methods

Pollen diffusates

Pollen extracts from *O. europaea*, *D. glomerata*, *C. sempervirens* and *P. sylvestris* were prepared by gently stirring 800 mg of pollen grains (Allergon, Ångelholm, Sweden) into 40 ml of 50 mM Tris–HCl pH 7.4 for 2 h at room temperature. This mixture was centrifuged for 10 min at 12 000 *g*. Pollen crude extracts were obtained after filtration (0.45 µm pore) of the supernatant. Protein concentration was determined by the BCA Protein Assay (Pierce, Rockford, IL, VA, USA) using BSA (Bovine serum albumin) as standard. Enzyme assays and cell-based experiments were performed applying the same volume of pollen crude extracts.

Gel electrophoresis

The SDS–PAGE system developed by Laemmli (16) was used to assess protein profile of crude extracts. Gels were stained with Coomassie Blue R-250 (Sigma-Aldrich, Madrid, Spain) or with silver staining (17). Prestained Precision Protein Standards (Bio-Rad, Amadora, Portugal) were used as molecular weight markers.

Zymography in semidenaturing condition

Gelatin zymogram was performed as previously described (10), followed by staining with Coomassie Brilliant Blue R-250.

2D-zymography

Pollen diffusates (5 ml) were concentrated to approximately 500 µl through ultrafiltration using a 30 kDa Centricon (Millipore, County Cork, Ireland). Concentrated diffusates containing approximately 100 µg of total protein were dissolved 1 : 1 in immobilized pH gradient (IPG) rehydration buffer without reduction agents (3% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); 0.2% (v/v) pH 3–10 carrier ampholines). Rehydration of IPG strips (pH 3–10, 7 cm; GE Healthcare, Uppsala, Sweden) was followed by isoelectric focusing through voltage linear gradient (250–4000 V). Immobilized pH gradient strips were equilibrated in SDS equilibration buffer (50 mM Tris–HCl pH 8.8, 30% glycerol, 2% SDS) for 20 min, after which proteins were separated by gelatin zymography (10).

Enzyme assays

Pollen proteolytic activity was assayed against several peptide substrates coupled to 7-amino-4-methylcoumarin (AMC). The enzyme reaction was initiated via the addition of 100 µM fluorogenic substrate to 200 µl of each pollen diffusate. The release of AMC was measured for 20 min at 37°C (see Data S1).

To characterize the pollen proteolytic activity, extracts were pretreated for 20 min with specific and endogenous protease inhibitors and metal ions (for effective concentrations, see Data S1). Enzyme activity was assayed, as described previously, using the preferred AMC substrate for each pollen extract.

Bioactive peptides degradation

For specificity studies, crude extracts were incubated for 3 and 12 h with several bioactive peptides at a protein ratio of 1 : 25 in 50 mM Tris–HCl, pH 7.4, at 37°C. Hydrolysis of the peptides was monitored by Multiple Reaction Monitoring mass spectrometry at the Proteomics Unit of the Center for Neuroscience and Cell Biology (see Data S1 and Fig. S1).

Cell culture

Calu-3 cells, from the American Type Culture Collection (Manassas, USA), were cultured at 37°C in 5% CO₂: 95% air in Dulbecco's modified Eagle's medium (Sigma-Aldrich,

Madrid, Spain) with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin.

Transepithelial permeability measurement

Calu-3 were grown using an air-interfaces culture as previously described (18). Cells were seeded at a density of 5×10^5 cells/cm² on the apical surface of a 12-transwell culture plate, with 0.4-µm polyester filters (Corning, Bath, UK). After 7 days, cells were washed with phosphate buffer saline (PBS), then incubated with serum-free medium in the basolateral chamber, and pollen diffusates were added to the apical chamber at 37°C. In control conditions, 50 mM Tris-HCl pH 7.4 was added to the apical chamber. Dose-response (0–5 mg/ml) and time-dependent (1–12 h) assays were performed. Cells were also exposed to pollen diffusates treated with 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) and different pollen combinations (volume 1 : 1). Afterwards, 50 µl of 100 µM rhodamine B isothiocyanate-dextran (RITC)-labelled Dextran 70S (Sigma) was added to the top chamber of the transwells. For the next 3 h, 50 µl of media samples was collected from the bottom compartments in 30 min intervals and analysed on a SpectraMax Gemini EM fluorescent plate reader (Molecular Devices, Sunnyvale, CA, USA) with an excitation wavelength of 530 nm and emission of 590 nm (SOFTMAX PRO v5 software, Molecular Devices, Sunnyvale, CA, USA). Epithelial permeability was calculated as the percentage of RITC-Dextran 70S in the bottom chamber solution over that in the top chamber solution per filter surface area (cm²) per unit of time (seconds).

Immunoblotting

Calu-3 cells were seeded at 5×10^5 cells/cm² in a 12-well culture plate (Corning). Upon confluency, cells were washed with serum-free medium and exposed for 3 h to the different stimuli. Control cells were exposed to serum-free culture medium. After incubation, adherent cells were washed twice with PBS containing protease inhibitors, scrapped to microfuge tubes and centrifuged at 12 000 g, for 5 min, at 4°C. Denaturing sample buffer [100 mM Tris-HCl/SDS; 5% (v/v) glycerol; 2% (w/v) SDS; 0.1 M DL-dithiothreitol (DTT)] was added to cell extracts. Denatured extracts were sonicated and centrifuged at 12 000 g, for 5 min. Following SDS-PAGE, proteins were electrophoretically transferred to polyvinylidene fluoride membranes (Millipore). Immunoblotting was performed as standard (19). Primary antibodies used were rabbit polyclonal anti-occludin, rabbit polyclonal anti-claudin-1, rabbit polyclonal anti-zonula occludens-1 (ZO-1) and mouse monoclonal anti-E-cadherin (Zymed Laboratories, Barcelona, Spain). Detection was performed by enhanced chemifluorescence technique (GE Healthcare, Buckinghamshire, UK). Immunoblots were normalized to β-actin (mouse monoclonal anti-actin; Sigma).

Immunocytochemistry

Calu-3 cells were grown to confluence in a 12-transwell plate using an air-interfaces culture, then washed with PBS and

incubated for 3 h with four different pollen extracts. After the different stimuli, cells were washed with PBS before fixation in methanol at –20°C for 10 min, then washed again in PBS. Cells were permeabilized with 0.3% (v/v) Triton X-100 in PBS for 10 min at 4°C and treated with blocking solution [10% (w/v) BSA in PBS] during 1 h at room temperature. Cells were stained with mouse monoclonal anti-occludin, rabbit polyclonal anti-claudin-1, mouse monoclonal anti-ZO-1 (Zymed Laboratories) or mouse monoclonal anti-E-cadherin (BD Biosciences, Franklin Lakes, NJ, USA), diluted in 3% (w/v) BSA/PBS, for 1 h at room temperature. After several washes in PBS, cells were labelled with secondary antibody Alexa-fluor 488, diluted in 3% (w/v) BSA/PBS, for 1 h at room temperature. Transwell membrane inserts were cut and mounted in DakoCytomation fluorescent mounting medium and examined using a Plan-ApoChromat 63× oil immersion objective under a confocal microscope (LSM 510 Meta; Carl Zeiss, Jena, Germany).

Statistical analysis

Data were expressed as means ± SEMs of three or more independent experiments. Statistical significance analysis was determined using 1-way ANOVA followed by Dunnett's for multiple comparisons with control conditions (in inhibition studies), or two-way ANOVA followed by Bonferroni post-test (in permeability and immunoblot assays). Differences were considered significant for *P*-values < 0.05.

Results

Pollen grains release high molecular weight proteases with major serine and/or aminopeptidase activity

Pollens were hydrated separately at a proportion of 20 mg grains/ml of 50 mM Tris-HCl buffer, pH 7.4, for 2 h. Protein concentration within *Olea* sp. and *Dactylis* sp. diffusates was 5 and 3 mg/ml, respectively, whereas low allergenic pollens liberated ten times less-soluble proteins: 0.5 mg/ml (*Cupressus* sp.) and 0.3 mg/ml (*Pinus* sp.). As different pollens clearly have a distinct ability to release their soluble components once hydrated, extracts variable protein concentration was maintained throughout all assays to reproduce what may occur *in vivo*.

To evaluate protein release and to assess the presence of proteolytic activity in the different pollens, each diffusate was analysed by SDS-PAGE (Fig. 1A) and gelatin zymography (Fig. 1B). All four pollen diffusates contained gelatin degrading enzymes, particularly high molecular weight proteases with mobility between 60 and 75 kDa, for *Olea* sp. diffusate, and between 75 and 100 kDa, for the other three extracts.

Pollen proteolytic activity was rapidly characterized through two-dimensional zymography, and we acknowledged low pI proteolytic spots in *Olea* sp., *Dactylis* sp. and *Pinus* sp. pollen diffusates (Fig. 1C). *Cupressus* sp. pollen exhibited a wider range of proteolytic spots, from acidic to basic pI (Fig. 1C).

To further characterize the pollen proteases, we performed enzymatic assays using AMC synthetic substrates and tested

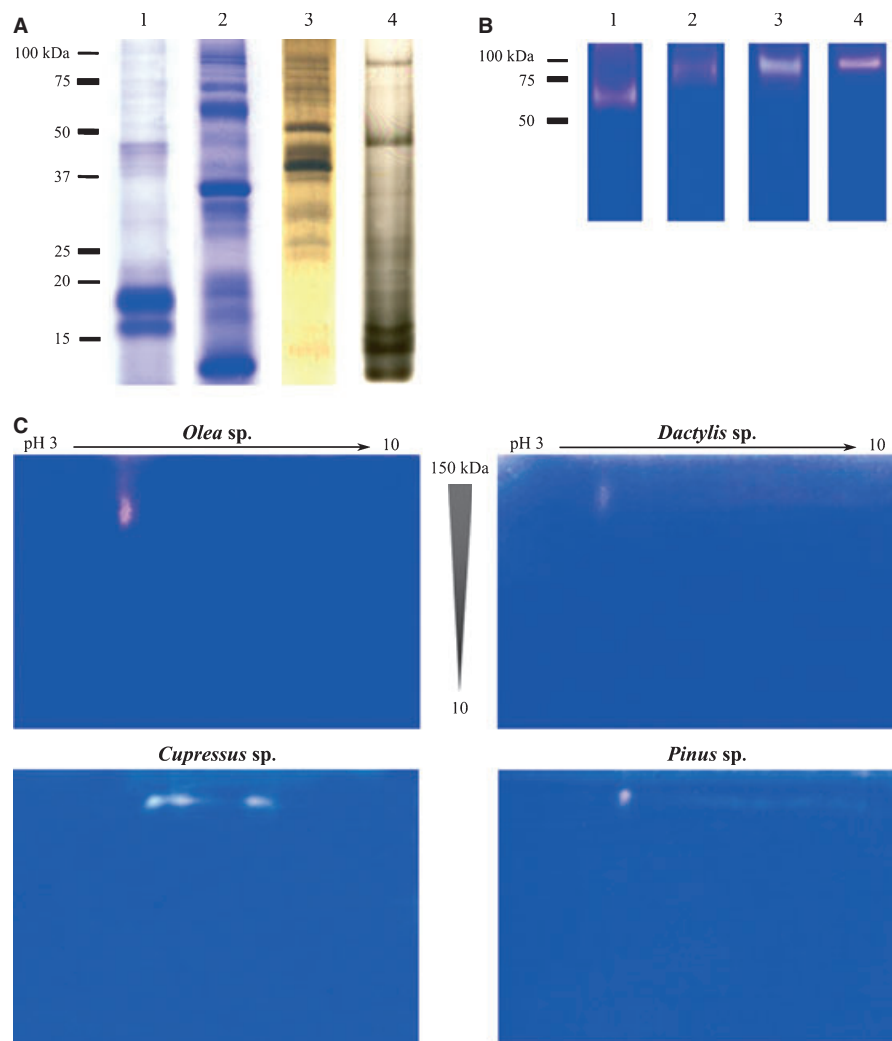


Figure 1 Protein and proteolytic profiles of pollen diffusates: Lane 1, *Olea europaea*; Lane 2, *Dactylis glomerata*; Lane 3, *Cupressus sempervirens*; Lane 4, *Pinus sylvestris*. (A) SDS-PAGE gels. Lane 1 and 2 were stained with Coomassie Blue R-250. Lane 3 and 4 were stained with silver nitrate because of the low amount of protein within these diffusates. (B) Gelatin zymography showing proteolytic

activity released by each pollen. (C) 2D-Zymography of pollen diffusates. Separation of proteins in the first dimension was achieved through isoelectric focusing in pH 3–10 immobilized pH gradient strips. Afterwards, proteins were separated according to molecular weight on a gelatin zymogram. Proteolytic spots were detected after Coomassie staining.

the action of several class-specific inhibitors, human endogenous protease inhibitors and divalent ions on pollen proteases activity (Fig. 2).

All pollen diffusates revealed proteolytic activity against various substrates. Phe-AMC was the preferred substrate for *Olea* sp., *Dactylis* sp., *Cupressus* sp. pollen diffusates, and Benzoyl-Arg-AMC for *Pinus* sp. pollen diffusate (Fig. 2A). Accordingly, these substrates were used in the evaluation of pollen proteases specificity (Fig. 2B). All crude extracts were partially or totally inhibited by AEBSF, ethylenediaminetetraacetic acid (EDTA) and Zn^{2+} . *Pinus* sp. activity was also completely suppressed by TLCK, a trypsin-like protease inhibitor.

Inhibition by AEBSF for all diffusates indicates they have preferential serine activity in this particular assay. The

complete inhibition of *Pinus* sp. activity by TLCK, along with its favourite substrate, suggests the presence of a trypsin-like serine protease in the extract. Furthermore, given that AEBSF, EDTA and Zn^{2+} have been proven to inhibit aminopeptidases to some extent (10, 20), it could indicate as well the presence of this type of enzymes in tested diffusates.

Interestingly, pollen proteolytic activity from all four diffusates was not inhibited by any of the three endogenous inhibitors tested in this assay [aprotinin, α 1-antitrypsin or secretory leukoprotease inhibitor (SLPI)] (Fig. 2B). In addition, incubation of aprotinin or α 1-antitrypsin with each pollen diffusate resulted in the degradation of the inhibitors (data not shown).

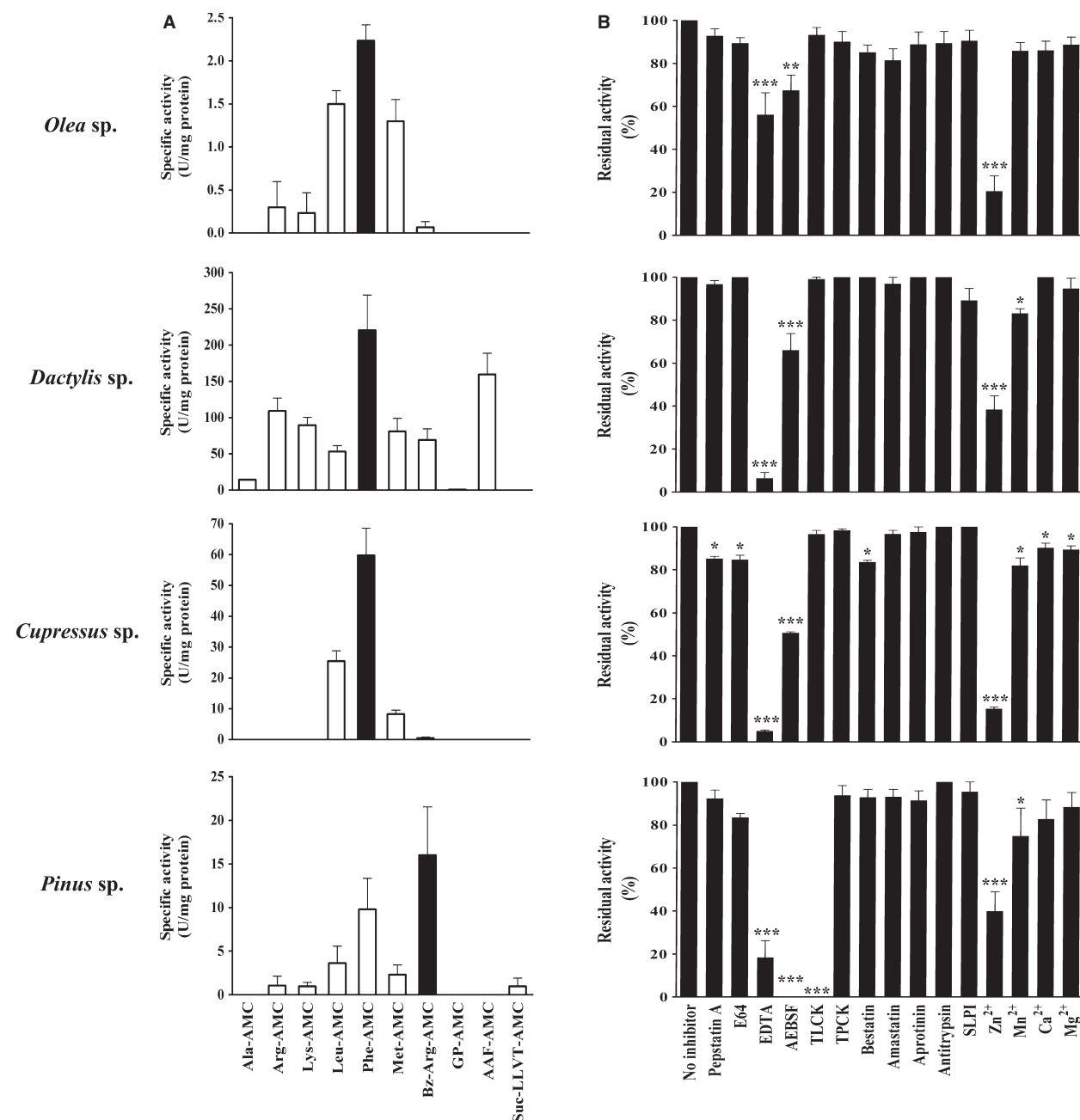


Figure 2 Specificity of pollen proteases from *Olea europaea*, *Dactylis glomerata*, *Cupressus sempervirens* and *Pinus sylvestris*. (A) Substrate specificity of pollen proteolytic activity. Whole extracts were incubated for 20 min at 37°C with 100 μ M peptide-7-amino-4-methylcoumarin (AMC). Black bars point out the preferred substrate for each pollen diffusate, which was also the substrate

used in the inhibition experiments. (B) Effect of class-specific inhibitors on pollen diffusates proteolytic activity. Results represent the percentage of residual activity when compared to control condition (without inhibitor). Dunnett's post-test: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ ($n = 3$). One unit of activity = pmol AMC released/min; Bz, benzoyl; Suc, succinyl.

Pollen diffusates induce the hydrolysis of bioactive peptides

Distinct studies reported the ability of pollen proteases to degrade several biopeptides involved in lung homeostasis (6–10). Thus, we investigated the specificity of cleavage of vasoactive and neurogenic mediators by different allergenic

pollen diffusates. According to Table 1, all crude extracts exhibited exopeptidase and oligopeptidase activity against the bioactive peptides tested. *Dactylis sp.* and *Cupressus sp.* crude extracts hydrolysed all three peptides. However, *Olea sp.* pollen proteases merely cleaved bradykinin that has an important role in vasodilation and increases vascular perme-

Table 1 Specificity of cleavage of bioactive peptides by pollen crude extracts

	Vasoactive intestinal peptide	Substance P	Bradykinin
<i>Olea</i> sp.	HSDAVFTDNYTRLRKQMA- -VKKYLNSILN	RPKPQQFFGLM	R↓PPGFSPF↓R
<i>Dactylis</i> sp.	HSDAVFTDNYTRLR↓KQ↓MA- -VKKKLNSILN	RPKPQ↓Q↓FF↓GLM	R↓P↓PGF↓SPFR
<i>Cupressus</i> sp.	HSDAVFT↓DNYTRLRKQ↓M↓A- -VKKYLN↓SIL↓N	RPKPQ↓Q↓FF↓GLM	R↓PPGFSPF↓R
<i>Pinus</i> sp.	HSDAVFTDN↓YTRLRK↓Q↓M↓A- -VK↓K↓YLN↓SILN	RPKPQ↓Q↓FF↓GLM	RPPGFSPFR

ability (21). Moreover, *Pinus* sp. pollen extract was able to degrade VIP, an anti-inflammatory neurotransmitter (22), and substance P, a proinflammatory peptide (23), with a very broad specificity.

Pollen diffusates here studied appear to have similar proteolytic specificity, particularly in the degradation of substance P by *Dactylis* sp., *Cupressus* sp. and *Pinus* sp., and in the

proteolysis of bradykinin by *Olea* sp. and *Cupressus* sp. (Table 1). Preferential cleavage sites occurred after arginine, glutamine or phenylalanine residues and are consistent with the specificity observed in the hydrolysis of AMC peptides (Fig. 2A). Pollen extracts cleavage specificity is also analogous to the aminopeptidase activity from *P. judaica* (10) and mesquite pollen (8).

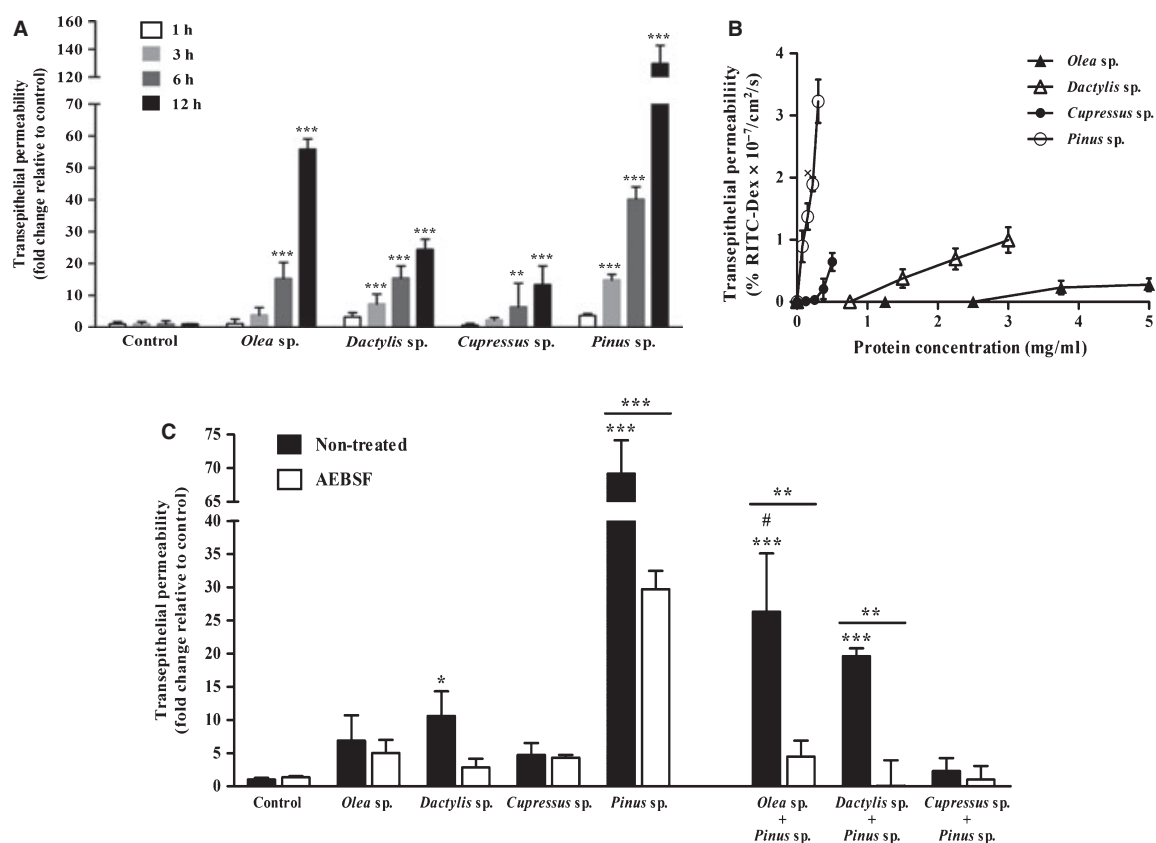


Figure 3 Effect of pollen proteases from *Olea europaea* (5 mg/ml), *Dactylis glomerata* (3 mg/ml), *Cupressus sempervirens* (0.5 mg/ml) and *Pinus sylvestris* (0.3 mg/ml), on the paracellular permeability of epithelial cells. Calu-3 cells were exposed to nondiluted pollen diffusates at different time-points (A); and to diffusates with distinct

protein concentrations for 3 h (B). Cells were also incubated with pollen diffusates treated with 1 mM AEBSF, as well as combinations of two pollen extracts (volume 1 : 1) (C). Bonferroni post-test: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; # $P < 0.01$ (compared to *Olea* sp.) ($n = 4$).

Pollen proteases increase epithelial permeability through disruption of intercellular complexes

Conducting airways are composed of epithelial cells that are connected by tight junctions and adherens junctions proteins (11). Mucosal DCs are separated from inhaled air by the epithelium barrier that acts as a molecular filter for antigens and pathogens. Hence, we studied the action of pollen proteolytic activity on the integrity of Calu-3, a human airway epithelial cell line (24). Cells were cultured at the air-liquid interface, which generates a more representative model of the airway epithelium (18).

All crude extracts induced an increase in transepithelial permeability in a time and dose-dependent manner, although *Pinus* sp. diffusate exhibited a more significant effect (Fig. 3 A,B). After 3-h stimuli, we observed that *Dactylis* sp. and *Pinus* sp. diffusates produced extremely significant effects, differing from *Olea* sp. and *Cupressus* extracts (Fig. 3A). The dose-response assay showed that *Pinus* sp. pollen produced a higher variation in permeability despite its lower protein concentration, meaning that the effect of pollen diffusates on the airway epithelium is not related to total protein levels, but to the amount or specificity of proteases released. Thus, from this point on, all experiments were performed using the highest protein concentration obtained for each extract and

3 h incubation periods (the lowest period with significant results).

To determine whether this effect could be related to pollen proteases, we preincubated pollen extracts with AEBSF, a serine protease inhibitor proven to inhibit all four pollen diffusates (Fig. 3C). Indeed, *in vitro* epithelial permeability caused by exposure to *Pinus* sp. pollen was partially reversed by AEBSF. On the contrary, the reduced effect induced by *Olea* sp., *Dactylis* sp. and *Cupressus* sp. diffusates was not significantly suppressed by AEBSF. As demonstrated in AMC assays (Fig. 2B), the inhibitor does not completely block their proteolytic activity which may explain its inefficacy under these conditions. Other common pollen protease inhibitors, such as EDTA and Zn^{2+} , were not tested in this assay because of their cytotoxic effects.

Interestingly, when combining *Pinus* sp. to *Olea* sp. and *Dactylis* sp. diffusates we noticed it had an adjuvant effect (Fig. 3C). This result is not observed when conjugating *Pinus* sp. and *Cupressus* sp. extract. Through 2D-Zymography (Fig. 1C) we acknowledged that *Cupressus* sp. pollen released a wider range of proteases which may interfere with *Pinus* sp. activity. In fact, the diminished transepithelial permeability induced by cypress pollen (alone or combined) may explain the reduced prevalence of allergy to this pollen although it has two allergens identified (13, 25).

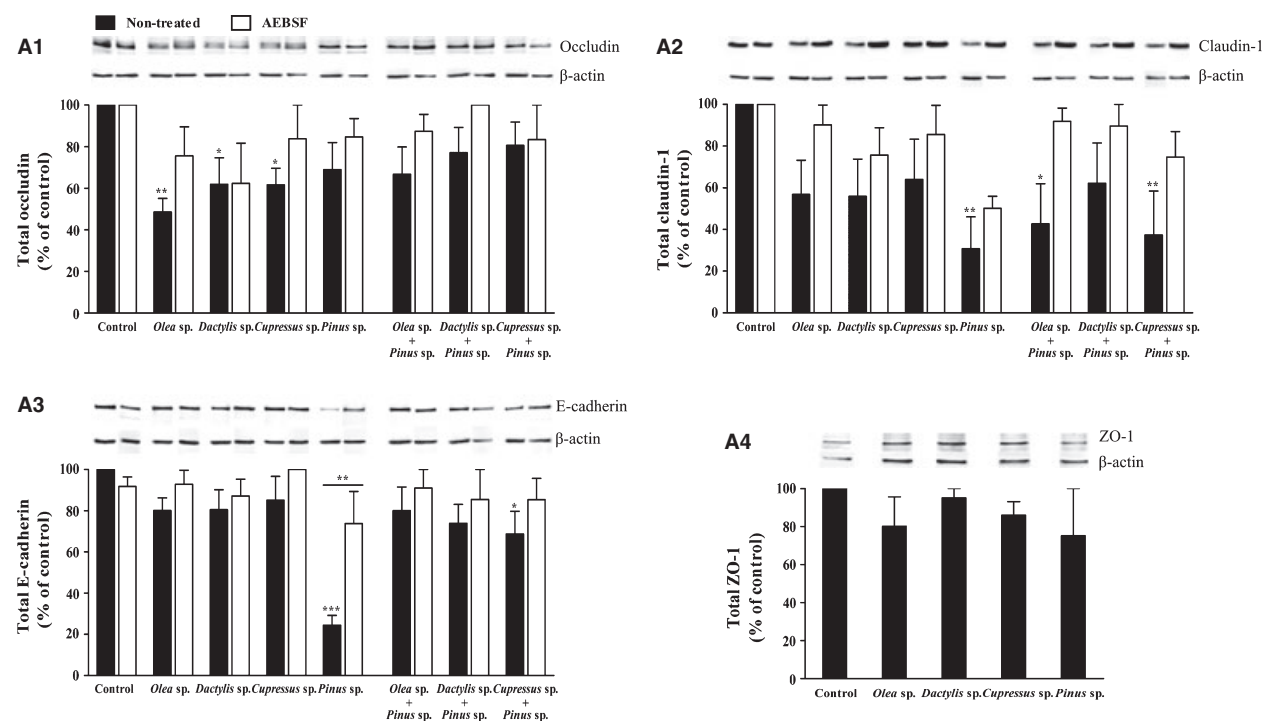


Figure 4 Effect of pollen diffusates on intercellular junctions integrity. Calu-3 cells were exposed for 3 h to pollen diffusates from *Olea europaea* (5 mg/ml), *Dactylis glomerata* (3 mg/ml), *Cupressus sempervirens* (0.5 mg/ml) or *Pinus sylvestris* pollen extract (0.3 mg/ml). Cells were also incubated with pollen diffusates treated with 1 mM AEBSF, as well as combinations of two pollen extracts (vol-

ume 1 : 1). Degradation of transmembrane proteins, occludin (A1), claudin-1 (A2), E-cadherin (A3); and cytosolic complex zonula occludens-1 (A4), was analysed by immunoblot. Representative blots are shown for each stimulus. Bonferroni post-test: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ ($n = 4$).

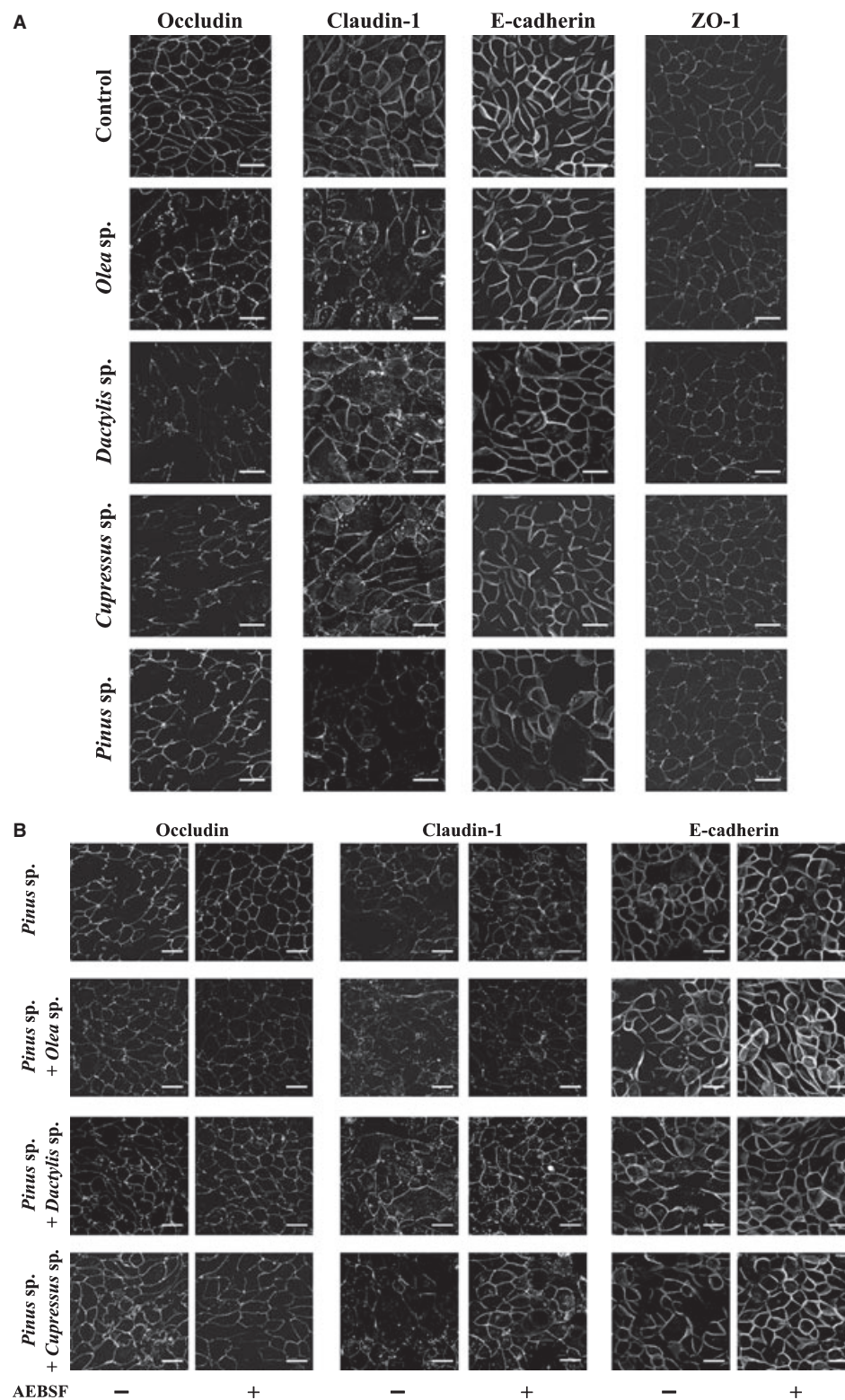


Figure 5 Distribution of intercellular adhesion proteins after exposure to pollen diffusates. Calu-3 were incubated for 3 h to pollen diffusates from *Olea europaea* (5 mg/ml), *Dactylis glomerata* (3 mg/ml), *Cupressus sempervirens* (0.5 mg/ml) or *Pinus sylvestris* pollen extract (0.3 mg/ml). Cells were also exposed to pollen diffusates

treated with 1 mM AEBSF, as well as combinations of two pollen extracts (volume 1 : 1). Intercellular junctions distribution was studied by immunofluorescence. Representative images are shown for each stimulus. Bar = 20 μ m.

The considerable increase in the epithelial permeability induced by pollen proteases, shown in Figure 3, is likely to arise from cleavage of intercellular complexes. Thus, we analysed the integrity of four junctional proteins, by immunoblot and immunofluorescence, after exposure of Calu-3 cells to different pollen extracts during 3 h (Figs 4 and 5). Immunoblot experiments suggest that occludin is degraded by *Olea* sp., *Dactylis* sp. and *Cupressus* sp. pollen proteases (Fig. 4A1), which is in accordance with the immunofluorescence images. In Figure 5A, control cells exhibit a continuous labelling of occludin at the surface, whereas treated cells have breaks in these continuous rings, show irregular labelling and a decrease on fluorescence intensity. *Pinus* sp. pollen had a greater impact on claudin-1 and, especially, on E-cadherin integrity (Figs 4A2,A3 and 5A). Furthermore, *Pinus* sp. pollen potentiated the activity of *Olea* sp. and *Cupressus* sp. diffusates on claudin-1 (Figs 4A2 and 5B), and also the effect of *Cupressus* sp. pollen on E-cadherin (Figs 4A3 and 5B). All degradations induced by pollen extracts were reversed after treatment with AEBSF (Figs 4 and 5B). Moreover, pollen diffusates had no effect on cytosolic protein ZO-1 (Figs 4A4 and 5B).

Discussion

The respiratory system is continuously exposed to a variety of environmental factors with potential to interact with several cell receptors, but it is still unclear how these features alter gene expression profile. Both endogenous and exogenous proteases have now been recognized to act through protease-activated receptors (PARs). Furthermore, airborne allergens should cross the epithelial barrier to be processed by DCs and trigger the allergic sensitization through IgE production. Several studies suggest that the presence of exogenous proteolytic activity might degrade endogenous proteins and help allergens reach subepithelial immune cells (26).

Pollen carries enzymes, along with allergens, that are released in the airways when grains are inhaled and hydrated. Pollen proteases, that have their primary role on pollen germination, possess a significant effect on allergic disorders (2–5, 10).

Very high concentrations of pollen solutes can be achieved when pollen grains and ambient air particulate matter are inhaled (50 mg/ml) (3, 27). In this study, we focused on four pollen species: two highly allergenic pollens (*Olea* sp and *Dactylis* sp.) and two pollen types with moderate to low allergenicity but with high airborne counts during the pollen season (*Cupressus* sp. and *Pinus* sp.) (1, 13–15). Pollen grains were hydrated *in vitro* to mimic the process that naturally occurs in the respiratory airways when an individual is exposed to high pollen counts. We used the same initial proportion (of pollen weight per volume of buffer) to prepare the four diffusates. However, protein concentration in the different pollen extracts ranged from 0.3 to 5 mg/ml, thus demonstrating pollen distinct ability to release proteins.

Characterization of pollen proteolytic activity showed evidence of high molecular weight serine proteases and/or aminopeptidases in all four extracts. Moreover, *Pinus* sp.

pollen appears to specifically liberate a trypsin-like serine protease. In fact, serine protease inhibitors have been proven to decrease other pollen proteolytic activities, such as, mesquite aminopeptidase (8) and trypsin-like serine protease (9), the aminopeptidase from *P. judaica* pollen (10), the ragweed arginine-specific peptidase (6) and endopeptidase (7), as well as Japanese cypress, white birch, *P. pratensis* pollen diffusates (12, 28).

In contrast, pollen proteases from the four tested pollens were not blocked by serine protease inhibitors (aprotinin, α 1-antitrypsin and SLPI) that usually protect the lower respiratory tract against endogenous proteases (Fig. 2). These inhibitors are likely to be hydrolysed by pollen proteases resulting in their inactivation (7, 10), indicating that pollen proteases can be active when released in the respiratory tract, and therefore be implicated in chronic inflammatory changes associated with allergic disorders.

Pollen proteases may be involved in neurogenic inflammation that plays a significant role in allergic diseases such as asthma (22, 23). We demonstrated pollen diffusates cause the hydrolysis of bioactive peptides released by nerve endings (Table 1) that *in vivo* can disturb the balance between the anti- and proinflammatory effects in the lung. This condition may exacerbate the bronchial hyperresponsiveness characteristic of asthma.

Other than neurogenic inflammation, our results imply that proteases from four pollens increase the paracellular permeability of the epithelium by disruption of transmembrane adhesion proteins: occludin (by *Olea* sp., *Dactylis* sp. and *Cupressus* sp.), claudin-1 and E-cadherin (by *Pinus* sp.) (Figs 4 and 5). This dual effect may be attributed to the distinct specificity observed in pollen extracts (Fig. 2B).

Trypsin-like serine proteases may be very effective on the loss of epithelial integrity, as *Pinus* sp. diffusate caused a more damaging effect in the integrity of transmembrane cell junction proteins, particularly E-cadherin. This transmembrane protein from adherens junctions is a critical determinant of the epithelial barrier function. Indeed, it has long been thought that E-cadherin contributes to the assembly of other specialized cell–cell junctions, notably elements of the apical junctional complex in epithelia (29).

Still, intercellular complexes degradation by pollen proteases appears to be directed towards proteins with extracellular transmembrane domains, as cytosolic ZO-1 was not displaced (Figs 4A4 and 5A). This result suggests that degradation of intercellular adhesions is direct. In the airway epithelium, exposed domains of transmembrane proteins must be more accessible to exogenous macromolecules.

The loss of effectiveness of the epithelium barrier can also occur by the activation of a receptor expressed in the apical surface of epithelial cells, such as PAR-2 (30). As described for mites and moulds (30–32), allergen proteases can trigger PAR-2 activation, possibly initiate the disassembly of junctional complexes and promote the allergic sensitization and inflammation.

It is clear that the respiratory system is exposed to a multitude of airborne particles, with allergenic and proteolytic content. We have demonstrated that different allergenic

pollen grains release proteases shown to be efficient in the hydrolysis of several substrates: gelatin, a collagen-derived protein; synthetic peptides; and lung neuropeptides. Pollen proteases had also a significant *in vitro* effect on the paracellular permeability of epithelial cells and induced major degradation of cell junction proteins: occludin, claudin-1 and E-cadherin. *In vivo*, this proteolytic activity can help allergens cross the epithelial barrier and contact with DCs, thus intensifying the immune response.

So far, primary focus has been given to proteases derived from high allergenic species (2–10, 12), but our work suggests that even less allergenic pollens are likely to be involved in the allergic sensitization and in chronic respiratory inflammation. These enzymes may explain the extent of respiratory symptoms in subjects with non-IgE mediated rhinitis or asthma that takes place at the peak of pollen season (33). Thus, identification and characterization of pollen proteases accountable for the disruption of intercellular complexes and bioactive peptides constitute important steps to come across a specific therapeutic target in the prevention and treatment of allergic disorders related to pollinosis.

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Conflict of interest

The authors declare that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article at <http://www.wileyonlinelibrary.com>:

Data S1. Supplementary Methods.

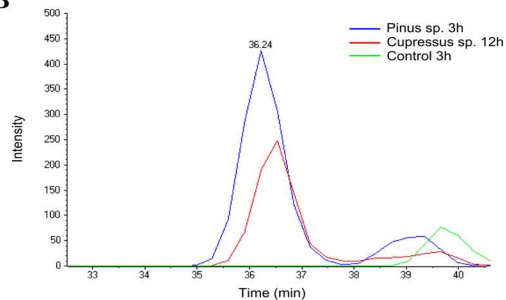
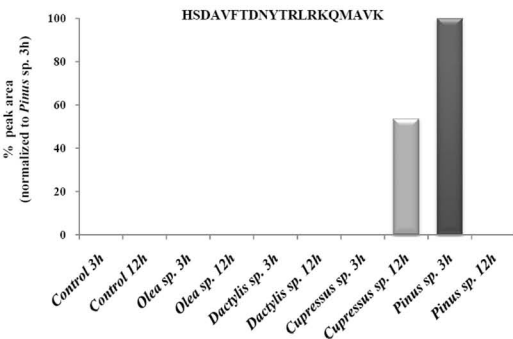
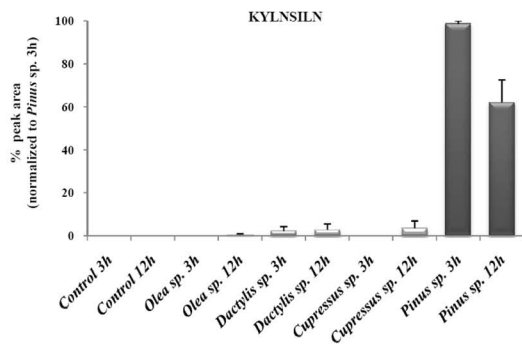
Figure S1. Determination of a cleavage site after incubation of VIP with *Pinus* sp. pollen proteases (HSDAVFTDNYTR-LRKQMAVK↓KYLNSILN).

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A

HSDAVFTDNYTRLRKQMAVKKYLNSILN

B**C****D**

Supplementary FIG 1

SUPPLEMENTARY METHODS

Enzyme assays

Pollen proteolytic activity was assayed against several peptide substrates coupled to 7-amino-4-methylcoumarin (AMC). The release of AMC was measured using a microplate fluorometer SpectraMAX-GeminiEM (Molecular Devices, Sunnyvale, USA) at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Reaction rates were measured as the slope of the progress curve obtained by continuous recording. AMC standard curve was used to determine the quantity of product released.

To characterize pollen proteolytic activity, extracts were pretreated with specific protease inhibitors that include: 1 μ M pepstatin A, 10 μ M L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), 10 mM EDTA, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 100 μ M N-p-tosyl-L-lysine chloromethyl ketone (TLCK), 100 μ M N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 10 μ M bestatin or 10 μ M amastatin. We also pretreated each pollen diffusate with several endogenous protease inhibitors (80 μ M aprotinin, 2 μ M α 1-antitrypsin or 10 μ g/mL of secretory leukoprotease inhibitor (SLPI)) and with metal ions (1 mM Zn^{2+} , 1 mM Mn^{2+} , 1 mM Ca^{2+} or 1 mM Mg^{2+}).

Bioactive peptides degradation

Multiple Reaction Monitoring (MRM) experiments were carried out as previously described,¹ with modifications, at the Proteomics Unit of the Center for Neuroscience and Cell Biology. The MIDAS™ Workflow Designer software was used to predict the most favorable Q1/Q3 transitions for all possible generated peptides upon digestion (this included considering 4 different charge states and at least 3 transitions per peptide per charge state). Digested peptides were analyzed on an Ultimate 3000 nanoLC system (Dionex). Peptides were

desalted on-line for 5 minutes using a trap column (300 μm i.d. x 5 mm C18 PepMap 100, 5 μm , 100 Å; Dionex) and resolved on a reverse phase capillary column (75 μm ID x 15 cm C18 PepMap 100, 3 μm , 100 Å; Dionex). A multiple step gradient from 15 to 100% acetonitrile in 0.1% formic acid over 33 minutes was used (flow rate of 250 nL/min). Mass spectrometry analysis was performed on a 4000 QTRAP® system using a NanoSpray® Source (Applied Biosystems/MDS Sciex).

MRM quantitative data was analyzed using MultiQuant™ software. Peak integrations were reviewed manually and transitions for analyte peptides were confirmed by the same retention time. Each peak area was determined to calculate relative peptide quantity and the results were plotted for each condition (Supplementary Fig 1). Cleavage specificity was assessed using the following criteria: relative peptide quantity after incubation of the peptide with pollen extracts for the shorter period (3 hours); number of transitions that produced quantitative data for the peptide; detection of the complementary peptide; and also consistency of protease cleavage specificity.

SUPPLEMENTARY REFERENCES

1. Silva RM et al. Critical roles for a genetic code alteration in the evolution of the genus *Candida*. EMBO J 2007;26:4555.

SUPPLEMENTARY FIG 1. Determination of a cleavage site after incubation of VIP with *Pinus* sp. pollen proteases (HSDAVFTDNYTRLRKQMAVK↓KYLNSILN).

A, VIP sequence. **B,** MRM chromatogram of transition 595.8/246.2 to detect HSDAVFTDNYTRLRKQMAVK peptide after incubation of VIP with *Pinus* sp. pollen diffusate for 3h or with *Cupressus* sp. pollen diffusate for 12h. Control condition corresponds to incubation of VIP in 50 mM TrisHCl, pH 7.4, for 3h. **C,** Relative peptide quantification (HSDAVFTDNYTRLRKQMAVK) for each condition, using data from one transition (595.8/246.2). **D,** Relative peptide quantification of the complementary peptide (KYLNSILN) for each condition, using data from six different transitions (Mean ± SEM).