

Original article

Purification of a novel aminopeptidase from the pollen of *Parietaria judaica* that alters epithelial integrity and degrades neuropeptides

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Background: *Parietaria judaica* pollen is a common cause of pollinosis in the Mediterranean area.

Objective: This study sought to purify and characterize the peptidase responsible for the majority of proteolytic activity present in the pollen extract of *P judaica*, and to investigate its contribution to the allergic response.

Methods: A series of chromatographic steps was applied to isolate the peptidase from *P judaica*'s pollen, and its biochemical properties were determined. Bioactive peptides present in the airways were incubated with the peptidase, and their degradation was visualized by direct protein sequencing. In addition, we measured the cellular detachment, by methylene blue binding assay, of an airway-derived epithelial cell line (A549) in the presence of the peptidase, and visualized, by Western blot, the degradation of proteins from intercellular junctions.

Results: We purified a 98-kDa peptidase from the pollen of *P judaica* that was classified as an aminopeptidase on the basis of its biochemical properties and internal amino acid sequence. The aminopeptidase was able to degrade bioactive peptides. Moreover, the aminopeptidase caused cellular detachment of A549 cell line and degradation of occludin and E-cadherin.

Conclusion: Our results suggest that the *P judaica* aminopeptidase can alter the integrity of the epithelium barrier by degrading occludin as well as E-cadherin. In addition, *P judaica* aminopeptidase can degrade bioactive peptides, which can exacerbate the overall bronchoconstrictive effect detected in asthmatic lungs.

Clinical implications: The novel aminopeptidase described here could constitute a relevant therapeutic target in the treatment of allergic disorders induced by the pollen of *P judaica*. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *Parietaria judaica*, aminopeptidase, VIP, substance P, angiotensin I, occludin, E-cadherin

The respiratory system is repetitively exposed to a wide variety of airborne particles, some of them with capacity to recruit and activate immunocompetent and inflammatory cells, which results in allergic diseases (bronchial asthma, allergic rhinitis, and atopic dermatitis).¹ Whereas the events that occur in established asthma are well understood, the process of allergen delivery that initiates all allergic diseases is unresolved.²

Airborne allergens derived from plant pollens are important triggers of allergic respiratory diseases.³ Besides the inflammatory effect of pollens as a result of their allergenic contents, the presence of proteolytic activity in pollen extracts from several species has been described,^{4,5} which may have an additional effect on the allergic response. Indeed, proteases released by a variety of allergenic pollens shown to be able to cause concentration-dependent detachment of murine airway epithelial cells from their substrate *in vitro*.⁴ Disruption of the epithelial barrier by proteases from pollen might facilitate transepithelial transport of proteins, which could promote sensitization as a result of increased access of allergenic proteins to subepithelial antigen-presenting dendritic cells.⁴ Moreover, Travis and colleagues⁶⁻⁹ have characterized the properties of several proteases released from ragweed and mesquite pollen. The isolated peptidases from those pollens were able to degrade bioactive peptides, such as substance P, vasoactive intestinal peptide (VIP), and angiotensin II, which are required for normal lung function. Thus, pollen peptidases could be involved in exacerbating the development of asthma by inactivating bioactive peptides that have ameliorating effects in maintaining lung airway homeostasis.

Parietaria pollen is one of the most common causes of pollinosis in the Mediterranean area¹⁰ and has also been described in other temperate climates of Central and Eastern Europe, Australia, and California.¹¹ *Parietaria* is a genus of dicotyledonous weeds belonging to the Urticaceae family, which is composed of several allergenic species. Immunologically, the most important species are *Parietaria judaica* and *Parietaria officinalis*. The major allergens of both species are small glycoproteins with

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Abbreviations used

APN: Aminopeptidase N

pNa: *p*-Nitroanilide

VIP: Vasoactive intestinal peptide

molecular weights ranging between 10 and 14 kDa, with high cross-reactivity. Sequence comparison suggests that the two *P judaica* major allergens belong to the nonspecific lipid transfer protein family,¹¹ which are classified in the pathogenesis-related protein families as PR-14.¹² Lipid transfer proteins are involved in defense mechanisms against pathogens and environmental stress, and are described as plant pan-allergens.¹³ Recently, we detected the presence of proteolytic activity in *P judaica* pollen extract. Therefore, to further characterize the proteolytic activity found in *P judaica* pollen extract, we fractionated the pollen extract and isolated the peptidase, Pj-peptidase, which accounts for the majority of the proteolytic activity present in this extract. We show that the Pj-peptidase is capable of degrading bioactive peptides (VIP, substance P, bradykinin, and angiotensin I) involved in the maintenance and recovery of the bronchomotor tone. Moreover, we prove that Pj-peptidase was also able to cause cellular detachment of airway epithelial cells in culture by degrading the tight-junction protein, occludin, and the adherent junction protein, E-cadherin. The damaging effect of Pj-peptidase on the airway epithelium is likely to promote an increased access of *P judaica* allergenic proteins to subepithelial antigen-presenting dendritic cells, and could therefore play a major role in potentiating the allergic response.

METHODS**Enzyme purification**

Pollen extract was prepared by stirring 4 g *P judaica* pollen (GE Healthcare Bio-Science, Uppsala, Sweden) into 20 mL 50 mM Tris-HCl pH 8.1 (buffer A) for 4 hours at 4°C. A crude extract was obtained by centrifugation of the mixture (10,000 rpm, 10 minutes, 4°C), with retention of supernatant. This crude extract was applied to DEAE-Sephacel ion exchange column (GE Healthcare Bio-Science) (1.5 × 4.5 cm, 8 mL) equilibrated with buffer A. After sample loading, the column was washed with the same buffer, followed by a one-step gradient with 1 mol/L NaCl in buffer A (buffer B), in a total volume of 40 mL. Fractions (3 mL) were collected and assayed for activity that cleaved the synthetic peptide substrate L-Ala-pNa. Those fractions containing activity were pooled (12 mL), and proteins were precipitated with 70% saturation of solid ammonium sulfate at 4°C. The precipitate obtained after centrifugation (10,000 rpm, 10 minutes, 4°C) was resuspended in 2.5 mL buffer A. The active solution was then applied to a HiLoad Superdex 200 gel filtration column (GE Healthcare Bio-Science) (1.6 × 60 cm, 120 mL) previously equilibrated with buffer A. The column was washed, at 1 mL/min, with the same buffer, and the collected fractions (4 mL) were assayed against L-Ala-pNa. Those fractions possessing activity were pooled (20 mL) and applied to a HitrapQ ion exchange column (GE

Healthcare Bio-Science) (0.7 × 2.5 cm, 1 mL) equilibrated with buffer A. The column was washed, at 1 mL/min, and the bound active enzyme was eluted with a linear gradient from 0 to 50% buffer B in 35 minutes. The active fractions were pooled (7 mL) and dialyzed overnight, at 4°C, against 2 L of 20 mM phosphate buffer pH 7.6 (buffer C). The dialyzed fraction was applied to an Arginine-Sepharose affinity column (GE Healthcare Bio-Science) (0.8 × 2 cm, 1 mL) previously equilibrated with buffer C. A linear gradient of 0 to 0.5 mol/L NaCl in buffer C was applied in a total volume of 20 mL, and the collected fractions were assayed for activity. Finally, the active fraction was concentrated to 1 mL using an Amicon-Centriprep YM-3 membrane (Millipore, Madrid, Spain).

Gel electrophoresis

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system developed by Laemmli¹⁴ was used to monitor enzyme purification and estimate the enzyme's molecular mass. Gels were stained with Coomassie Blue R-250 (Sigma-Aldrich, Madrid, Spain). The prestained Precision Protein Standards (Bio-Rad, Amadora, Portugal) were used as a standard.

Zymogram in semidenaturing condition

Gelatin zymogram was performed by copolymerization of 1 mg/mL of the substrate into the separating gel of SDS-PAGE (12% polyacrylamide). Samples were applied without reduction or boiling (dilution 1:1 with 125 mM Tris-HCl, pH 6.8, containing 4% SDS and 20% glycerol) and separated with 20 mA/gel at 4°C. SDS was removed by shaking in 2 changes of 50 mM Tris-HCl, pH 7.5, containing 2.5% (vol/vol) Triton X-100 (Calbiochem, Darmstadt, Germany) for 15 minutes. After rinsing in distilled H₂O, activity was then revealed by incubation overnight at 37°C in 50 mM Tris-HCl, pH 7.5, without shaking, followed by staining in Coomassie Brilliant Blue R-250.

Enzyme assays

For routine assays during purification of the enzyme, the activity of the peptidase was measured spectrophotometrically at 405 nm with the synthetic peptide L-Ala-pNa (1 mmol/L final concentration) in 1 mL 50 mM Tris-HCl, pH 8.1, at 30°C. The same conditions were used for determination of optimal pH and temperature. The amidolytic activity with several substrates was determined in 1 mL of the same buffer and at the same temperature. In inhibitory studies, the enzyme was preincubated with inhibitors for 30 minutes at 30°C before the substrate (L-Ala-pNa) was added, and the enzyme activity was measured as described.

Protein concentration was determined by the Bio-Rad Protein Assay with BSA as the standard.

Kinetics parameter for L-alpNa, V_{max} and K_m , were determined by using substrate concentration ranging from 0.068 to 2 mM with the final concentration of the enzyme 2 µg/mL in 50 mM Tris-HCl, pH 8.1, at 30°C. The activity was measured spectrophotometrically at 405 nm, as described. V_{max} and K_m values were determined by using linear regression analysis.

Mass-spectrometric analysis

For amino acid sequence analysis, the purified protein was resolved by SDS-PAGE and excised from the gel. The protein band was analyzed by MS-MS spectrometry at the Centro de Genómica e Proteómica, Facultad de Farmacia, Universidad Complutense de Madrid, Spain.

Neuroactive peptides degradation

For specificity studies, the purified enzyme was incubated from 10 to 20 minutes with several bioactive peptides at enzyme:substrate ratio of 1:100 in 50 mM Tris-HCl, pH 8.1, at 30°C. The peptide

TABLE I. Purification of Pj-peptidase

Fractionation step	Total protein (mg)	Total activity (μmol/min)	Specific activity* (U/mg prot.)	Yield (%)	Purification factor
Pollen extract (4 g)	151.20	24.80	0.16	100	
DEAE-Sephacel	51.72	10.80	0.21	43.5	1.3
Ammonium sulfate precipitation	39.23	7.30	0.19	29.4	1.2
Superdex 200	15.80	7.30	0.46	29.4	2.9
HitrapQ	1.47	3.10	2.10	12.5	13.1
Arginine-Sepharose	0.29	1.00	4.88	4.0	30.5

*The assay was performed at 30°C in 50 mM Tris-HCl, pH 8.1, with 1 mM, final concentration, of L-Ala-pNa. 1 unit of activity = μmol pNa released/min.

fragments were directly used for N-terminal sequence determination by automated Edman degradation on an Applied Biosystems 473A protein sequencer (GE Healthcare Bio-Science).

Cell culture and incubation conditions

A549 cells, a human alveolar type II epithelium-like cell line (American Type Culture Collection, Manassas, Va), were cultured at 37°C in 5% CO₂: 95% air in F-12K culture medium modified by Ham Kainghn (Sigma, Madrid, Spain) with 10% heat-inactivated FBS (Gibco, Barcelona, Spain) and 50 μg/mL gentamicin (Gibco). A549 cells were seeded at 3.9×10^5 cells/mL in a 96-well culture plate, and 24 hours after seeding, cells were washed with serum-free medium and incubated for 24 hours with different concentrations of pollen extract or Pj-peptidase.

Cellular detachment assay

Cellular detachment was quantified by methylene blue binding assay.⁴ After incubation for 24 hours, cells were gently washed with PBS and fixed 10 minutes with 10% phosphate-buffered formalin. Cells remaining attached were stained with 1% methylene blue in 0.01 mol/L borate buffer, pH 8.5, for 10 minutes at room temperature and washed 5 times with borate buffer. The bond dye was subsequently eluted with a 1:1 mixture of absolute ethanol and 0.1 M HCl, and absorbance of the wells was read at 620 nm. The percentage of adherent cells was calculated relative to the absorbance of wells incubated in basal medium. Cells incubated with 10 μg/mL trypsin were used as a positive control.

Immunoblotting

A549 cells were seeded at 3.9×10^5 cells/mL in a 6-well culture plate, and 24 hours after seeding, cells were washed with serum-free medium and incubated for 24 hours with pollen extract or Pj-peptidase, in the absence or presence of the inhibitor bestatin (0.01 mmol/L). After incubation, adherent cells were washed twice with PBS containing protease inhibitors, scraped to Eppendorf tubes, and centrifuged. Boiling sample buffer (Tris-Bicine, 100 mM; urea, 8 mol/L; SDS, 2%; 2-β-mercaptoethanol, 2%; bromophenol blue 0.002%) was added to the cell extract. After SDS-PAGE, proteins were transferred electrophoretically to PVDF membranes (Roche Applied Sciences, Penzburg, Germany). Immunoblotting was performed as standard. Primary antibodies used were mouse polyclonal antioccludin, rabbit polyclonal anti-ZO-1, mouse monoclonal anti-E-cadherin, and rabbit monoclonal anticlaudin (Zymed Laboratories, Barcelona, Spain). Detection was by enhanced chemifluorescence technique (GE Healthcare Bio-Science).

Statistics

Data were expressed as means ± SEMs of the indicated number of experiments. Statistical significance analysis was determined by using 1-way ANOVA followed by Dunnett for multiple comparisons

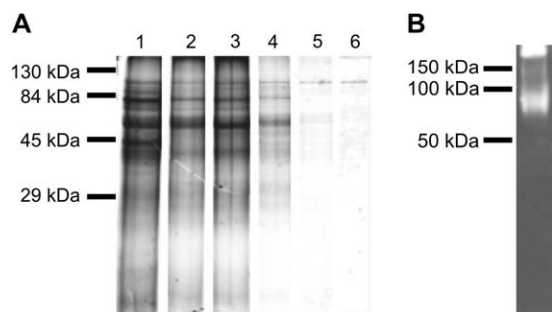


FIG 1. A, SDS-PAGE of samples from different stages of the Pj-peptidase purification. Lane 1, pollen extract; lane 2, DEAE-Sephacel eluate; lane 3, ammonium sulfate precipitate; lane 4, Superdex 200 eluate; lane 5, Hitrap Q eluate; lane 6, arginine-Sepharose eluate. B, Gelatin zymography showing the proteolytic activity of the purified Pj-peptidase. The gels were stained with Coomassie Blue R-250.

with the control or the impaired 2-tailed Student *t* test. The differences were considered significant for *P* values <.05.

RESULTS

Enzyme purification

The peptidase from *P. judaica* (Pj-peptidase) was readily liberated from the pollen grains by gentle stirring with buffer at 4°C with a maximum activity within 4 hours. Because previous results obtained with the *P. judaica* pollen extract showed that the amidolytic activity against L-Ala-pNa was the major proteolytic activity present in the pollen extracts, we used this substrate in the enzymatic assays during the purification procedure. As shown in Table I, several steps were required to purify 290 μg (30.5-fold) of the Pj-peptidase from 4 g pollen with a final yield of 4%. The use of a DEAE-Sephacel ion exchange chromatography step at the beginning of the purification procedure was essential for the partial elimination of pigments and some contaminating proteins. The subsequent gel filtration step was responsible for removing the totality of the pigments and proteins of low molecular weight. Finally, the affinity step with arginine-Sepharose resulted in a purified protein with an apparent molecular mass of 98 kDa (Fig 1, A). A single degradation band was produced when this last fraction of the purification protocol was analyzed by gelatin zymography (Fig 1, B). The

TABLE II. Amidolytic activity of Pj-peptidase

Substrate	Concentration (mmol/L)	Specific activity* (U/mg protein)
L-Ala-pNa	1	7.0
L-Arg-pNa	1	2.7
L-Val-pNa	1	0
L-Leu-pNa	1	0
L-Ala-Ala-Ala-pNa	1	0
N-Suc-Ala-Ala-Val-pNa	1	0
N-Suc-Ala-Ala-Pro-Phe-pNa	1	0
Bz-Phe-Val-Arg-pNa	0.5	0
Bz-Arg-pNa	1	0

Bz, Benzoyl; Suc, succinyl.

*The assay was performed at 30°C in 50 mM Tris-HCl, pH 8.1.

1 unit of activity = $\mu\text{mol pNa}$ released/min.

SDS-PAGE and zymogram analysis revealed that the purified enzyme migrates as a single band with 98 kDa, in agreement with the data obtained by gel filtration chromatography.

Biochemical characterization of Pj-peptidase

The purified Pj-peptidase was tested for its amidolytic activity on several nitroanilides synthetic peptides. As shown in Table II, L-Ala-pNa was the preferred substrate (and thus was used in further assays), with the next best being L-Arg-pNa. Longer peptides were inefficient as substrates. An amino-terminus blocking group (succinyl or benzoyl groups) abolished activity. The results indicate that the amidolytic activity of the Pj-peptidase requires a free amino group at the NH_2 -terminus of a substrate, and it prefers to cleave basic (arginine) and small (alanine) amino acids at the amino terminus of substrates. Using the amidolytic activity assays with L-Ala-pNa, it was found that the enzyme has broad pH stability from pH 7.5 to 9, with optimal activity at pH 9. The activity of the purified enzyme was optimal at 40°C, although 40% of the enzyme activity was observed at 25°C. No enzyme activity was detected at temperature higher than 60°C. The enzyme showed no appreciable loss of activity when frozen at -20°C, although frequent cycles of freezing and thawing caused some loss of activity.

Table III summarizes the effect of several class-specific inhibitors on the Pj-peptidase activity. Neither pepstatin, an aspartic specific-inhibitor, nor E-64, a cysteinic inhibitor, could inhibit Pj-peptidase activity. Pj-peptidase activity was inhibited by high concentration of the serine protease inhibitor PMSF. The TPCK inhibitor, an inhibitor of chymotrypsin-like enzymes, had a more significant inhibitor effect on Pj-activity than TLCK, an inhibitor of trypsin-like enzymes. The Pj-peptidase activity was almost completely inhibited by bestatin and amastatin, two aminopeptidase inhibitors, which supports the conclusion, driven from activity assays (Table II), that the Pj-peptidase has exopeptidase activity. Chelating agents 1,10-phenanthroline and EDTA had an inhibitory effect on Pj-peptidase activity, indicating that a metal ion can be involved in the enzymatic activity. The presence of Zn^{2+} and Mn^{2+} caused complete inhibition at 1 mM,

TABLE III. Effect of class-specific inhibitors on the activity of Pj-peptidase*

Inhibitor	Class	Concentration (mM)	Residual activity
Pepstatin	Aspartic	0.001	100
E-64	Cysteine	0.01	100
Chymostatin	Cysteine/serine	0.1	39
PMSF	Serine	1	6
TPCK	Serine	0.1	8
	chymotrypsin-like		
TLCK	Serine trypsin-like	0.1	64
Bestatin	Aminopeptidase	0.01	2
Amastatin	Aminopeptidase	0.01	1.5
EDTA	Metalloprotease	5	1.5
1,10-Phenanthroline	Metalloprotease	10	3
$\alpha 1$ -Antitrypsin	Serine	0.002	100
Aprotinin	Serine	0.08	80
Ca^{2+}		1	76
Mg^{2+}		1	71
Mn^{2+}		1	0
Zn^{2+}		1	2

E-64, L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane; PMSF, phenylmethanesulfonyl fluoride; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; TPCK, N-p-tosyl-L-phenylalanine chloromethyl ketone.

*Results are for 30-minute incubation at 30°C in 50 mM Tris-HCl, pH 8.1, with 1 mM L-Ala-pNa as substrate.

whereas slight inhibitory effects were observed in the presence of Ca^{2+} and Mg^{2+} . Of note, the Pj-peptidase activity was not inhibited by the $\alpha 1$ -antitrypsin inhibitor or aprotinin, both human endogenous inhibitors. The Pj-peptidase showed a typical Michaelis-Menten kinetic activity against L-Ala-pNa (data not shown), and the kinetics activity parameters, V_{max} and K_m , determined for this substrate were $5.8 \mu\text{molmin}^{-1}\text{mg}^{-1}$ and 0.4 mM, respectively.

Internal sequence comparison

Because the amino-terminal of the Pj-peptidase was blocked, N-terminal sequencing was not possible, and therefore the enzyme was analyzed by MS-MS spectrometry. The internal sequence obtained, SSGNFCTQCEAEGFR, was submitted to BLAST from NCBI database, and it showed 100% identity with a putative aminopeptidase N (APN) from *Arabidopsis thaliana* and 93% identity with a putative APN from *Oryza sativa*. High homology was obtained with APN from microorganisms (Fig 2). APNs belong to the M1 family, which forms part of the MA clan of metalloproteases. The internal sequence of the peptidase from mesquite pollen⁸ has a similar homology with APN's sequences from *A thaliana* and *O sativa* and microorganisms (Fig 2), although the sequences from Pj-peptidase and mesquite peptidase did not overlap.

Enzymatic hydrolysis of regulatory neuropeptides by Pj-peptidase

Many neuropeptides localized in the airways have been shown to have potent effects on bronchomotor tone,

Pj-peptidase	(-)	SSGNFCTQCEAEGFR -----
<i>A. thaliana</i> PepN	(116)	SSGNFCTQCEAEGFRKITFYQDRPDIMAKYTCRVEGDKTLYPVLLSN
<i>II_{mes}</i> peptidase	(-)	-----KITFYQDRPDIMARYTLKIEADKYLYPVLLSN
<i>O. sativa</i> APE	(115)	STGNFCTQCEAEGFRKITVFGDRPDVMATVTCRIEADKYLYPYLLSN
<i>E. coli</i> PepN	(112)	SGDALCTQCEAEGFRHITYYLDPRDVLARFTTKIADKTKYPFLLSN
<i>P. syringae</i> APE	(118)	SSGMFCTQCEAEGFRKITVYLDPRDVMSTFTTTLSDAKQNFPIILLSN

FIG 2. Internal amino acid sequence of Pj-peptidase and alignment with aminopeptidases sequences from plant and microorganisms. Accession numbers of amino acid sequences are as follows: AAN72085 (*A. thaliana*), XP_483801 (*O. sativa*), AAC74018 (*E. coli*), and NP793562 (*P. syringae*). The *II_{mes}* peptidase (from mesquite pollen) was obtained from Matherson and Travis.⁸

mucus production, airway microvascular leakage, and inflammatory and immune cells. Several studies reported the capability of pollen proteases to degrade these proteins and peptides involved in normal respiratory function.⁶⁻⁹ For these reasons, we investigated whether Pj-peptidase could efficiently degrade essential neuropeptides *in vitro*.

As reported in Table IV, the Pj-peptidase was able to degrade two neuropeptides, substance P and VIP, which are essential for regulating bronchomotor tone. In addition, the Pj-peptidase was also able to degrade angiotensin I and bradykinin, both peptides involved in the inflammatory response. The Pj-peptidase showed exopeptidase and oligopeptidase activity against bioactive peptides. The Pj-peptidase had a broad specificity, able to degrade bioactive peptides with arginine as well as aspartate and histidine at the amino-terminal. Angiotensin I was sequentially degraded from its amino-terminus. Internal cleavage was also detected in the biopeptides VIP, substance P, and bradykinin. Taken together, the results indicate that the Pj-peptidase behaves as an aminoendopeptidase. Such classification is not a new concept, because Matheson and Travis⁸ described a peptidase (Ilmes) from mesquite pollen that showed an aminopeptidase and oligopeptidase activity similar to the one described here. In addition, *Escherichia coli* PepN,¹⁵ cathepsin H,¹⁶ and many peptidyl dipeptidases (eg, cathepsin B¹⁷) were described as aminoendopeptidases.

Pj-peptidase induces cell detachment in the pulmonary epithelial cell line A549 by degrading occludin, a tight-junction protein

The epithelial lining barrier of the airway is a developmentally specialized barrier that must be crossed for inhaled allergens to encounter dendritic antigen-presenting cells.¹⁸ Interestingly, previous studies showed that solutes released by a variety of allergenic pollens were able to cause cellular detachment of murine airway epithelial cells from their substratum *in vitro*.⁴

Therefore, we compared the cellular detachment induced by exposure of A549 cell monolayers to *P. judaica* pollen extract or to Pj-peptidase (Fig 3, A and B). In both conditions, we observed cell detachment in a concentration-dependent manner.

Intercellular junctions, such as tight-junctions and adherent junctions, between adjacent epithelial cells form intercellular seals. Maintenance of these seals is vital to maintaining the integrity of epithelium barrier in the airways. To go deeper into the molecular mechanisms

TABLE IV. Specificity of cleavage of bioactive peptides by Pj-peptidase

Peptide	Sequence
VIP	H- ¹ -S-D-A-V-F-T-D-N-Y-T-R-L-R-K-Q-M- ¹ -A-V-K-K-Y-L-N- ¹ -S-I-L-N
Substance P	R-P-K-P-Q- ¹ -Q-F-F- ¹ -G-L-M
Angiotensin I	D- ¹ -R- ¹ -V-Y-I-H-P-F-H-L
Bradykinin	R- ¹ -P-P-G-F- ¹ -S-P-F-R

underlying the cellular detachment observed in the presence of Pj-peptidase, whole cell extracts of A549 cells were immunoblotted to ascertain whether cell junction proteins underwent proteolysis after cell exposure to Pj-peptidase (Fig 3, C). Occludin, a transmembrane protein present in tight-junctions, was degraded after treatment of A549 cells with Pj-peptidase (0.4 mg/mL). This effect was inhibited in the presence of bestatin inhibitor, indicating that occludin degradation was mediated by the proteolytic activity of Pj-peptidase. Moreover, the addition of *P. judaica* pollen extract (5 mg/mL) to A549 cells also led to occludin degradation, and the presence of bestatin prevented this effect. Similar results were obtained for E-cadherin, although with a lesser extent of degradation (Fig 3, D). In contrast, exposure of A549 cells to Pj-peptidase or *P. judaica* pollen extract did not result in degradation of ZO-1 or claudin-1, other proteins present in the tight-junction assemblies (data not shown). These results indicate that the *P. judaica* pollen could promote cell detachment, possibly by degradation of specific cell adhesion proteins.

DISCUSSION

Allergic diseases such as bronchial asthma, allergic rhinitis, and atopic dermatitis are dramatically increasing all over the world. Pollen is known to be a significant triggering factor for the development of both allergic responses and asthma.

In addition to allergens, pollens release a variety of enzymes on hydration, including proteases. In the current report, we describe the isolation of a peptidase from the pollen of *P. judaica* that could play a significant role in the pathophysiology mechanisms that occur during pollinosis. On the basis of the inhibition by bestatin and amastatin (Table III), as well as its preference for Ala residue at the amino terminus of the substrate (Table II), the enzyme characterized in this work is a 98-kDa alanyl

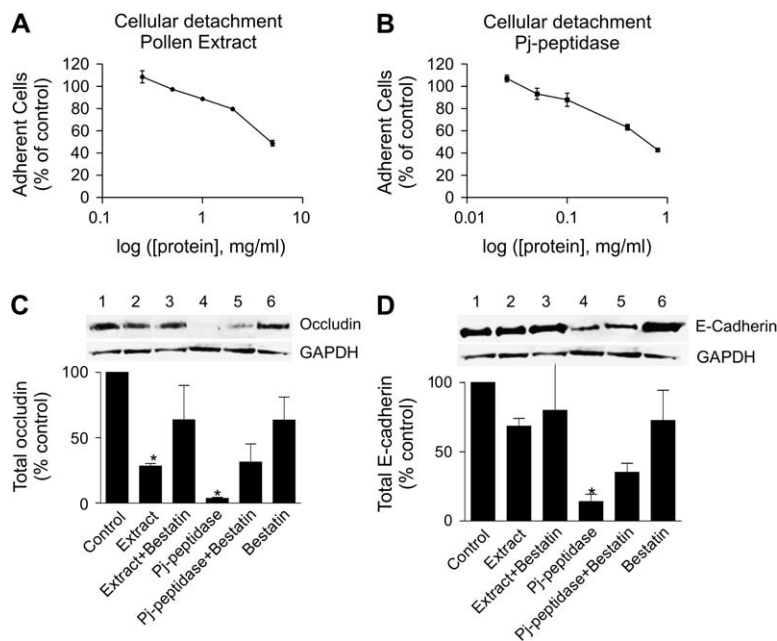


FIG 3. Dose-dependent effect of *P judaica* pollen extract (**A**) and Pj-peptidase (**B**) on cell detachment of A549 cell line. Data are means ($\pm$ SEMs) of two different experiments performed in duplicates. **C and D**, Immunoblots of junctional proteins from A549 cell monolayers treated with *P judaica* pollen extract or Pj-peptidase. Occludin (**C**) and E-cadherin (**D**) were degraded after exposure of A549 cells to *P judaica* pollen extract (lane 2) as well as Pj-peptidase (lane 4). In both cases, this effect was reverted by the addition of bestatin (lanes 3 and 5). Representative Western blots are shown. At least triplicates were run for each condition, using independent preparations, as indicated. Data were normalized and expressed as percentage control. Data are plotted as the means $\pm$ SEMs. * $P < .05$; Bonferroni test. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

aminopeptidase. Indeed, the internal amino-acid sequence obtained from mass spectrometry revealed high homology with the zinc-containing APNs (alanyl aminopeptidase) both from plants and microorganisms (Fig 3), including an APN purified from mesquite pollen (peptidase Ilmes).⁸ APNs belong to the M1 family, which forms part of the MA clan of metallopeptidases. Interesting, Pj-peptidase activity is not blocked by α 1-antitrypsin, the major endogenous protease inhibitor, and therefore it seems likely that the peptidase might be active when released from pollen in the respiratory tract, playing a role in the development of allergic response.

An important pathological feature in asthma is airway inflammation. A mechanism linking chronic airway inflammation to deranged physiological function is altered enzymatic inactivation of lung peptides. Two such peptides essential for regulating airway response are substance P and VIP.^{19,20} We demonstrated that Pj-peptidase is able to degrade, *in vitro*, both peptides, which may promote an imbalance in the regulation of the bronchomotor tone during the allergic response. In addition, angiotensin I, a potent vasoconstrictor, was cleaved and inactivated by the peptidase, which could also contribute to the overall vasodilation observed in asthmatic lungs. Travis and colleagues⁶⁻⁹ also isolated several proteases from ragweed and ambrosia pollen, which can degrade bioactive peptides, and were proposed to contribute significantly to the pathological effects observed in allergic-related asthma.

Previous studies indicated that proteases released from a variety of allergenic pollens might be of biological importance because they are able to cause detachment of murine airway epithelial cells. Moreover, the detachment activity of pollen proteases was not inhibited by endogenous antiproteases.⁴ Using the A549 cell line, a human alveolar type II epithelium-like cell line, we showed that *P judaica* pollen extract exposure promotes cellular detachment. It has been reported that 20% to 25% of the pollen mass is released rapidly on hydration, and therefore very high concentrations (as much as 50 mg/mL) of pollen solutes are likely to be achieved when pollen grains are deposited on mucosal surfaces.⁵ Therefore, the release of Pj-peptidase from pollen may cause a denudation of the airway epithelium, which might promote sensitization as a result of increased access of allergenic proteins to the subepithelial antigen-presenting dendritic cells. In addition, tissue damage by the Pj-peptidase could directly induce an inflammatory response.

The structural integrity of the epithelium depends, in part, on various types of cell-cell and cell-matrix adhesions, such as tight-junctions and adherent junctions.²¹ Exposure of A549 cells to *P judaica* pollen extract and, more specifically, to Pj-peptidase led to degradation of two proteins involved in cell-cell adhesion, occludin, and E-cadherin. Occludin is a transmembrane protein thought to contribute to the adhesive seals of tight-junctions and regulate paracellular pathways. Thus, cleavage of occludin may contribute to tight-junction disassembly

and increased permeability of the airway epithelium. Other tight-junction proteins, claudin-1 and ZO-1, were not degraded after exposure of A549 monolayer to pollen extract or purified Pj-peptidase, indicating that occludin degradation is specific. E-cadherin, a transmembrane protein of the adherent-junctions, also underwent proteolysis, but to a much smaller extent than occludin. Because adherent-junctions are located beneath tight-junctions,²² the smaller extent of proteolysis may arise simply because degradation of tight-junctions is an essential prerequisite for diffusional access of Pj-peptidase to adherent-junctions. Alternatively, the extracellular domains of E-cadherin may be less suitable substrates than the extracellular loops of occludin. Further investigations will be necessary to identify the sites of cleavage in occludin and E-cadherin by Pj-peptidase. Many clinically important allergens, such as house dust mite allergens and some fungal allergens, have been shown to possess enzymatic activity, which seems to interfere with the epithelium barrier. Indeed, Der p 1, the major mite allergen, disrupts epithelial tight-junctions, increases epithelial permeability, and may facilitate allergen delivery.^{2,21,23} 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.^{24,25} Therefore, it is proposed that by enabling allergens to breach the integrity of the airway epithelial barrier, proteolytic activity plays an adjuvant proallergic role influencing immunogenicity.²⁶

Taken together, these results suggest that Pj-peptidase, released from *P. judaica* pollen, may be involved in the initial steps of sensitization by disruption of epithelium barrier, which may enhance the delivery of allergenic protein to dendritic cells. Moreover, Pj-peptidase may have a role in the degradation of bioactive peptides involved in the maintenance of bronchial function. Therefore, pollen proteases may have significant importance in allergic rhinitis, asthma, and other respiratory complications associated with pollen exposure, not only through the production of IgE-type antibodies but also through a direct effect on the respiratory tract itself.

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