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 serial 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 2006;118:878-84.)

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 colleagues[6-9] 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 area[10] 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
*p*Na: *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-*p*Na. 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-*p*Na. 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-Centripep YM-3 membrane (Millipore, Madrid, Spain).

Gel electrophoresis

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system developed by Laemmli[14] 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 semidenaturating 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 H_2O, 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-*p*Na (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-*p*Na) 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-ala-*p*Na, 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 Genomica e Proteomica, Faculdad 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