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# IgE-binding trypsin inhibitors in plant pollen extracts

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**Abstract.** In an attempt to access the possible role of protease-antiprotease mechanisms of non-immune defence in pollinosis, only low levels of trypsin-, kallikrein- or plasmin-like proteinases could be detected in aqueous pollen extracts. In contrast, several pollen species displayed appreciable trypsin inhibitory activity, e.g. *Parietaria*, *Olea*, *Ambrosia*, *Rumex*, *Chenopodium*, *Holcus* and *Poa* spp. These proteins of the serpin family of anti-proteinases were found to bind specific IgE-antibodies from the serum of hay fever patients. As examples, the IgE-binding trypsin inhibitors from the pollen of *Parietaria judaica* and *Ambrosia elatior* were purified and characterized as acidic proteins with pI 4.2 and a molecular weight of 20–24 kDa.
**Key words.** Allergens; proteases; inhibitors; pollen.

It has often been pointed out that the potent allergens causing immediate-type human allergic disease may not simply act as foreign antigens inducing IgE and IgG antibodies, but may have additional biological or biochemical properties. Nevertheless, such possible auxiliary properties have only sporadically been investigated[1–4]. Recent studies have classified predominant allergens of the dust mite *D. pteronyssinus* as serine- and cysteine-proteases[5]. Some of the inhalant allergens in the flour of wheat and barley are now known to be members of the family of α-amylase and trypsin inhibitors[6]. The intimate association of allergenic properties with non-immunological biological activity is especially interesting in the case of allergens belonging to the group of protein hydrolases or to the families of anti-proteinases. Contact of the mucous membranes with such biologically active allergens may conceivably disturb the local proteinase–antiproteinase equilibrium, thereby inducing local irritation and an influx of cells of the immune system. Such allergens would then be capable of triggering the ensuing antigen-specific immunological response by acting as non-specific activators or adjuvants, especially in predisposed, so-called 'atopic' people[1,7].

With respect to the airborne pollens of allergenic plants little information is available on biochemical activities possibly associated with the predominant allergenic components. A relationship has been considered between the acid phosphatase or leucine aminopeptidase activities of pollen extracts with the binding properties for specific IgE-antibodies in the serum of pollinosis patients[8,9]. However, no systematic studies have so far been published concerning the possible relationship between the allergenicity of pollen proteins and proteinase or proteinase-inhibitor activities.

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## Materials and methods

Pure, dried pollen granules were purchased from Biopol Laboratories, Spokane, USA. The powders were exhaustively defatted with diethylether in a Soxhlet apparatus and dried in air prior to extraction with distilled water for 2 h at room temperature. The centrifuged aqueous extracts were dialysed in the cold against distilled water from Visking tubing (cut-off 10 kDa) and the non-dialysable retentates were dried by lyophilisation.

Further purification of trypsin inhibitor proteins was carried out by a series of steps involving molecular sieving on Sephadex® G100 columns and preparative isoelectric focussing in a Rotofor™ IEF cell (Biorad Laboratories, Hercules, USA). In a typical experiment, 30 mg samples of the lyophilised non-dialysable proteins of *Parietaria judaica* pollen in phosphate-buffered saline (PBS) were applied to a 100 × 2.6 cm column of Sephadex® G-100 and eluted with PBS. Effluent fractions were analysed for trypsin inhibitory activity. The inhibitor pool from 3 separate runs was collected and the purified protein was recovered by dialysis (membrane cut-off 10 kDa) against distilled water and lyophilisation. The purified inhibitor protein was then further fractionated by preparative isoelectric focussing in physiological salt solution using the Rotofor™ IEF system. Analysis showed that the trypsin inhibitors focussed in the compartment of pH 4.2–4.5. The purified trypsin inhibitor was collected from the corresponding cell, dialysed and dried by lyophilisation.

Proteinase inhibition was monitored using bovine pancreatic trypsin and the chromogenic tripeptide substrate for serine proteases, H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide-diHCl (S-2288, Kabi-Pharmacia, Sweden), in Tris-HCl buffer pH 8.4. The assay was carried out in the wells of microtiter plates, whereby 50 µl of bovine pancreatic trypsin (500 ng/ml) and 50 µl of the chro-

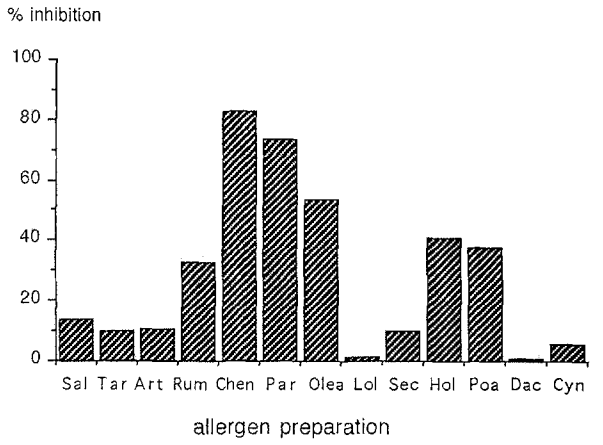


Figure 1. Screening of the soluble proteins (2.5 mg/ml) of various pollen species for inhibition of bovine trypsin, 7.7 BAEE-units/ml, using S-2288 as the chromogenic tripeptide substrate. Abbreviations: Sal (*Salsola kali*), Tar (*Taraxacum officinalis*), Art (*Artemisia vulgaris*), Rum (*Rumex acetosella*), Chen (*Chenopodium album*), Par (*Parietaria judaica*), Olea (*Olea europaea*), Lol (*Lolium perenne*), Sec (*Secale cereale*), Hol (*Holcus lanatus*), Poa (*Poa pratensis*), Dac (*Dactylis glomerata*), Cyn (*Cynodon dactylon*).

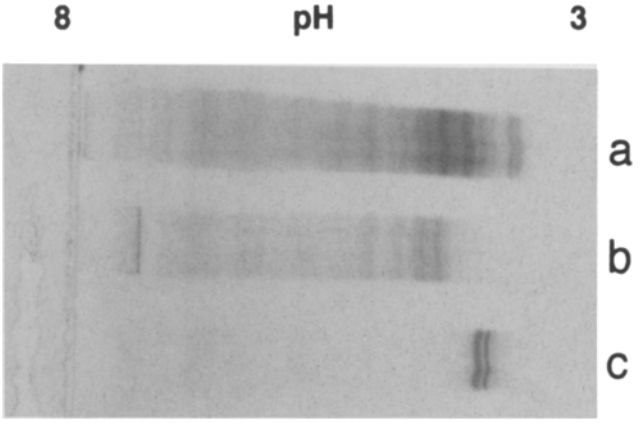


Figure 2. Analytical isoelectric focusing in polyacrylamide gel of *a* total proteins from the pollen of *Parietaria judaica* L., *b* the same preparation depleted of trypsin inhibitor, *c* the doublet of the isolated trypsin inhibitor proteins. Stain: Coomassie Brilliant Blue.

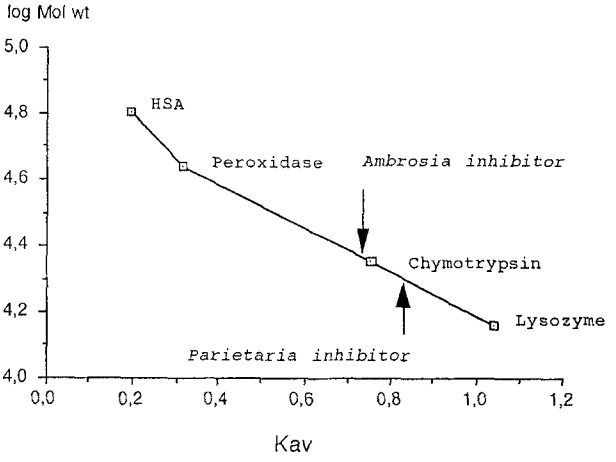


Figure 3. Estimation of the molecular size of the trypsin inhibitors from *Ambrosia elatior* and *Parietaria judaica* by molecular sieving on a 75 × 2.6 cm calibrated column of Sephadex G 100 in phosphate-buffered saline.

mogenic substate S-2288 (1.5 mM) in Tris-HCl buffer pH 8.4 were incubated for 30 min at 37 °C in the presence or absence of 50 µl of a dilution series of dialysed unfractionated pollen proteins or their purified subfractions. The reaction was terminated by the addition of 50 µl 50% acetic acid and the absorbance was read at 410 nm in a microplate reader.

Binding of specific IgE-antibody was determined by a RAST (RadioAllergoSorbent test) inhibition assay, using a series of dilutions of pollen protein allergens or chromatographic subfractions pre-incubated with a pool of sera of specifically allergic pollinosis patients. Residual specific IgE-antibody was then captured on cellulose discs to which the respective pollen proteins had been chemically coupled using cyanogen bromide, followed by incubation with peroxidase-labelled monoclonal anti-IgE (Ingenasa, Madrid, Spain). Bound peroxidase enzyme activity was finally determined by incubation with tetramethylbenzidine and hydrogen peroxide for 30 min at room temperature. The reaction was stopped with 2 N sulphuric acid, aliquots of the solutions were transferred to the wells of microtiter plates and the colour was read in a microplate reader at 450 nm.

## Results

Using specific chromogenic tripeptide substrates for trypsin (S-2288, Kabi-Pharmacia), tissue kallikrein (S-2266) or plasmin (S-2251) in direct assays we found only very low proteinase-specific amidolytic activity in most of the clinically relevant allergenic pollen preparations. In contrast, appreciable trypsin inhibitory activity activity could be demonstrated in extracts of a variety of pollen species. In the initial experiments, the water-soluble non-dialysable proteins ($M > 10$ kDa) from a number of allergenic pollens from different species were screened for the presence of trypsin inhibitors. The results for some of the well-known allergenic pollens are collectively shown in figure 1.

On the basis of these data, the trypsin inhibitors in the pollen of *Parietaria judaica* and *Ambrosia elatior* were chosen for more detailed investigation. The inhibitor proteins were isolated by a series of purification steps outlined above. The starting material and the intermediate and final trypsin inhibitor fractions were examined for binding of specific IgE-antibody using a pool serum of patients with clinical hay fever due to *Parietaria* sensitivity. The trypsin inhibitor from *Parietaria* pollen comprised 0.46% of the dry weight of the soluble and dialysed pollen proteins. The trypsin inhibitory activity increased from 0.21 units/mg to 35.0 units/mg (purification factor PF = 166), bringing the recovery of

activity to 76%. The IgE-binding potency for 50% inhibition increased by a factor PF of 3.8. The recovery of activity on the basis of IgE-binding was only 1.8%. Similar data were obtained from the inhibitor from *Ambrosia elatior*.

Upon analytical isoelectrofocussing in polyacrylamide gel in the pH-range 3–10 (1% Ampholines®, Biorad, USA) the purified inhibitor preparations demonstrated only two detectable and closely associated protein bands, which focussed at pI 4.2 (fig. 2).

The molecular size of the trypsin inhibitors was established by gel filtration on calibrated columns of Sephadex G 100, as exemplified in figure 3 for the *Ambrosia* and *Parietaria* species. By interpolation, the molecular weight of the inhibitors was estimated at 24 kDa and 20 kDa for the *Ambrosia* and *Parietaria* inhibitors, respectively.

## Discussion

In this investigation several plant pollens known to cause pollinosis (or 'hay fever') in humans have been found to contain inhibitors for bovine trypsin. The isolation and identification studies carried out on the pollen species of *Ambrosia elatior* and *Parientaria judaica*, as examples of strongly sensitizing agents, demonstrate that the molecular size and isoelectric points of these serine protease inhibitors are in agreement with those on record for many other plant or animals trypsin inhibitors of the serpin family[10]. As far as we are aware, proteinase inhibitors have not so far been reported in plant pollen, with the exception of a recently described cystatin proteinase inhibitor in the pollen of *Ambrosia artemisiifolia*[11]. It could also be shown that the trypsin inhibitors, which comprise less than 1% of the total extractable pollen proteins, are recognized as allergens by specific IgE-antibodies in the serum of specifically sensitized patients with pollinosis. However, in view of the discrepancy between the recoveries of activity of trypsin inhibition and the specific IgE-antibody binding, it appears that these allergenic inhibitor proteins comprise only a small proportion of the multiple pool of IgE-binding antigens known to occur in plant pollen extracts. It may nevertheless be speculated that their antiproteinase nature confers biological properties on the antigen molecule which may qualify these allergens as possible factors in the initiation or modulation of the allergic reaction, e.g. by blocking mast cell tryptase[12] or by interacting with activated proteinases of the contact phase- or complement-cascades.

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