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Purification and Characterization of a Novel Endopeptidase in Ragweed (Ambrosia artemisiifolia) Pollen

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Ragweed (Ambrosia artemisiifolia), the major cause of late summer hay fever (allergic rhinitis) in the United States and Canada, is clinically the most important source of the seasonal aeroallergens. A novel endopeptidase was extracted from the pollen of this plant and purified by a series of column chromatographic steps. It has a molecular mass of 82 kDa according to gel filtration and SDS-polyacrylamide gel electrophoresis and a pH optimum near 9.0, and its activity is unaffected by chelating or reducing agents. A 17-amin acid amino-terminal sequence of this protein showed no similarity with any other proteases. The enzyme was inhibited by diisopropyl fluorophosphate, a general serine class inhibitor, and more specifically N-p-tosyl-L-phenylalanine chloromethyl ketone, a chymotrypsin-like proteinase inhibitor. Various synthetic substrates were efficiently cleaved with a strong preference for Phe in the P1 and P3 position and Pro in the P2 position. This specificity was confirmed through inhibition studies with both peptidyl chloromethyl ketone and organophosphate inhibitors. In addition to synthetic substrates, the neuropeptides, vasoactive intestinal peptide and substance P, which are required for normalized lung functions, were also rapidly hydrolyzed. Activity toward protein substrates was not detected with the exception of the inactivation of α-1-proteinase inhibitor, which occurred through cleavage within the reactive site loop. These results indicate that the purified enzyme is a novel endopeptidase, which may be involved in both the degradation of neuropeptides and the inactivation of protective proteinase inhibitors during pollen-initiated allergic reactions.

Pollen grains contain a variety of proteins that are required in the fertilization of plants. Ragweed pollen, which is known to contain a complex mixture of over 60 different proteins, many of which are antigenic and, in some individuals, have the potential of being allergenic (1, 2), is perhaps the most clinically important, since it is the major cause of late summer hay fever in the United States and Canada (3). Exposure to moisture solubilizes these pollen proteins, several of which have been identified as powerful antigens and allergens that elicit IgE production in individuals who suffer from the symptoms of inhalant allergy (4). Furthermore, many have been found to have potential enzymatic activities that could alter normal homeostatic pathways (5).

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As asthma and allergic rhinitis are examples of closely related diseases that are difficult to distinguish, and the specific ways that these diseases are related have not yet been clearly defined (6). In the latter case, this occurs by pollen being deposited on the mucous membranes of the upper respiratory tract during inhalation. Upon contact, mucous liquid solubilizes pollen proteins, which are rapidly released and penetrate the mucous tissues. The mucous membranes of allergic patients contain high concentrations of mast cells, which contain IgE antibodies attached to their surfaces. Pollen allergens quickly complex with these antibodies, and the complexes activate enzymes that cause the release of mediators, including histamine, from these cells. Histamine induces allergic symptoms through the dilatation of blood capillaries, contraction of nasal and bronchial muscles, constriction of nasal and bronchial passages, and the hypersecretion of watery nasal fluids and from mucous membranes.

An important pathological feature of asthma is airway inflammation (7, 8). A mechanism linking chronic airway inflammation to deranged physiological function is altered enzymatic inactivation of lung neuropeptides. Two such peptides essential for regulating airway response are substance P and vasoactive intestinal peptide (VIP)1 (9–12), each of which has been proposed to have a major role in the neuroregulation of airway secretion, vascular permeability, and bronchomotor tone (13). Substance P, a potent bronchoconstrictor, which can cause degranulation of mast cells, is released from sensory neurons innervating airway epithelium, glands, blood vessels, and smooth muscle. Previous experimental data suggest that release of this neuropeptide from sensory nerve terminals may be a mechanism of neurogenic inflammation (14). VIP causes relaxation of human bronchial and vascular smooth muscle (15) and has been localized to efferent autonomic neurons (16). It is believed to be responsible for mediation of nonadrenergic smooth muscle relaxation in the lung and gut (17, 18). Evidence suggests that lungs with chronic allergic inflammation are more sensitive to the contractile effects of substance P and less sensitive to the relaxant effects of VIP (19). Therefore, disruption in VIP-mediated neural control may be considered a possible mechanism of exaggerated bronchoconstrictor responses characteristic of asthma (20).

1 The abbreviations used are: VIP, vasoactive intestinal peptide; α-1-PI, α-1-proteinase inhibitor; PVDF, polyvinylidene difluoride; DFP, di-isopropyl fluorophosphate; TPCK, N-p-tosyl-L-phenylalanine chloromethyl ketone; TLCK, N-p-tosyl-L-lysine chloromethyl ketone; FPLC, fast protein liquid chromatography; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis; serpin, serine protease inhibitor; bis-Tris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)-propane-1,3-diol; CK, chloromethyl ketone; Bz, benzoyl; Z or Cbz, benzoyloxycarbonyl; Suc, succinyl; Boc, t-butoxy carbonyl; pNA, p-nitroanilide.
The present study was initiated in an attempt to isolate and characterize pollen proteases, which might specifically degrade proteins or peptides involved in normal respiratory function. As a result of this investigation a novel serine endopeptidase from short ragweed (Ambrosia artemisiifolia) pollen was purified, and it was shown to degrade both VIP and substance P. In addition, it was observed that this peptidase was able to inactivate α-1-proteinase inhibitor (α-1-PI), a primary regulator of neutrophil elastase (21). This is particularly important within the lung, where its major function is to control tissue degradation. Thus, inactivation of this inhibitor may add to the complications of proteolytic degradation of the neuropeptides involved in airway inflammation and the altered physiological function that is characteristic of asthma caused by pollen exposure.

**EXPERIMENTAL PROCEDURES**

**Materials**

Common/short ragweed (A. artemisiifolia) was obtained from Miles Allergy Products (Spokane, WA) and polyvinyl difluoride (PVDF) membranes from Bio-Rad, respectively. Dioxopropyl fluorophosphate (DFP), leupeptin, and 3,4-dichloroisocoumarin, were from Calbiochem. Antipain, aprotinin, iodoacetamide, lime bean trypsin inhibitor, soybean trypsin inhibitor, N-succ-Ala-Ala-Arg-NA, N-Suc-Ala-Val-Ala-pNA, N-Suc-Ala-Val-Ala-pNA, N-Suc-Ala-Ala-Pro-Phe-pNA, N-Suc-Ala-Ala-Pro-Val-pNA, N-p-tosyl-l-lysine chloromethyl ketone (TLCK), N-p-tosyl-l-phenylalanine chloromethyl ketone (TPCK), substance P, and VIP were obtained from Sigma. E-64 (trans-eoxypseudcinnyl-l-leucyl-amido-4-quinidine) butane), EDTA, and pepstatin were from Boehringer Mannheim, omuicomic acid was from Worthington, and [3H]DFP was from DuPont NEN. Rabbit polyclonal antihuman α-1-proteinase inhibitor was a kind gift provided by Athens Research and Technology (Athens, GA). All substrates and inhibitors not listed were a kind gift provided by Dr. James Powers (Georgia Institute of Technology, Atlanta, GA).

**Methods**

**Enzyme Extraction and Enzyme Purification**—Pollen extracts were prepared by stirring 100 g of pollen into 1 liter of 10 mM ammonium bicarbonate, pH 8, for 24 h at 4 °C. A crude extract was obtained by centrifugation of the mixture (27,500 × g, 20 min, 4 °C), with retention of the supernatant. This solution was fractionated between 30 and 70% saturation with solid ammonium sulfate, and the precipitate obtained after centrifugation (27,500 × g, 20 min, 4 °C) was resuspended in 100 ml of 20 mM Bis-Tris-HCl, pH 6.5 (buffer A), followed by dialysis at 4 °C against the same buffer with three changes every 6 h. The dialyzed solution (117 ml) was applied to a DEAE-Sephadex ion exchange column (2.5 × 50 cm, 246 ml) equilibrated with buffer A. After application, the column was washed with the same buffer, and 23-ml fractions were collected until the A280 baseline fell below 0.05. All fractions were assayed for activity that cleaved the synthetic peptide substrate N-Suc-Ala-Ala-Pro-Phe-pNA.

The active pooled fall-through fractions (272 ml) were applied to a Cibacron blue Sepharose CL-6B affinity column (2.5 × 30 cm, 147 ml), previously equilibrated with buffer A. The column was then washed with buffer until the A280 base line fell below 0.025, followed by a linear gradient from 0 to 500 mM NaCl in buffer A in a total volume of 500 ml. Fractions (17 ml) were collected, and those containing activity were pooled (338 ml) and dialyzed 4 °C against 30% ammonium sulfate in buffer A with three changes every 2 h. The dialyzed fraction was applied to a phenyl-Sepharose CL-4B hydrophobic interaction column (1.5 × 30 cm, 53 ml), previously equilibrated with 30% ammonium sulfate in buffer A and washed in equilibration buffer until the A280 base line fell to zero. A gradient of 30–0% ammonium sulfate in buffer A was applied in a total volume of 300 ml, and the collected fractions (9 ml) were assayed against N-Suc-Ala-Ala-Pro-Phe-pNA. Those fractions possessing activity were pooled (106 ml) and concentrated to 8 ml by ultrafiltration using an Amicon PM-10 membrane. This material was applied to a Sephadex G-200 Superfine column (1.6 × 190 cm, 187 ml), equilibrated with 150 mM NaCl in buffer A, and the fractions (3 ml) containing activity were pooled (15 ml) and dialyzed at 4 °C against 50 mM sodium acetate, 5 mM CaCl2, pH 5.0 (buffer B), with three changes every 3 h. The dialyzed sample was applied to a Mono S HR 5/5 FPLC column (Pharmacia Biotech Inc.) and equilibrated with buffer B, and the column was washed at 1 ml/min until the base line stabilized near zero. Using a flow rate of 1 ml/min, the bound active enzyme was eluted in 15 min using a linear 0–125 mM NaCl gradient. Final purification to remove any minor contaminants was achieved by applying the active fractions to a TSK-GEL G300SW gel filtration column (TosoHaas Corp.) equilibrated with 200 mM NaCl in buffer A and run at 2 ml/min.

**Enzyme Assays**—Enzyme purification was monitored by amidolytic activity at 405 nm using the substrate N-Suc-Ala-Ala-Pro-Phe-pNA (4 μM), in 0.2 M Tris-HCl, pH 9.0. General proteolytic activity was measured using 10% (wt/vol) azocasein, described by Barrell and Kirschke (22). Electrophoresis and Autoradiography—Purification was monitored by Tricine SDS-PAGE (23) using a Tris-HCl/Tricine buffer system. The enzyme was radiolabeled using [3H]DDFF for sequence confirmation. 17 μCi/μg of protein was incubated for 30 min at 25 °C followed by a 30-min incubation with 10 μg cold DFPS. SDS-PAGE, was performed, and the gel was exposed for 96 h to x-ray film. The radiolabeled protein was blotted to a PVDF membrane for sequencing.

**Inactivation Studies of α-1-PI**—The inactivation of α-1-PI, indicated by a shift in the molecular mass of native α-1-PI, and the subsequent observation of cleavage products were performed using Laemmli SDS-PAGE with a 15% separating gel and the Laemmli buffer system (24). The inactivation of α-1-PI was performed at an E:1 ratio of 1:1000, and the shift in molecular mass determined by Western blotting. Rabbit polyclonal antihuman α-1-proteinase inhibitor was kindly provided by Athens Research and Technology, and Western blotting was performed according to the protocols of Transduction Laboratories. Antigen-antibody complexes were visualized by enhanced chemiluminescence.

**Sequence and Amino Acid Analysis**—Following SDS-PAGE, the native and radiolabeled enzyme, as well as fragmented α-1-PI, were each blotted to a PVDF membrane as described by Matsudaira (25). Sequence analysis was performed with an Applied Biosystems Procise Protein sequencer using the program designed by the manufacturer. For the analysis of peptide fragments from enzyme-treated polypeptide hormones, individual samples were hydrolyzed and subjected to amino acid analysis with the use of an Applied Biosystems 420A Derivatizer analyzer.

**Enzyme Specificity and Kinetics**—For specificity studies, substrates were incubated at an enzyme:substrate molar ratio of 1:1000 in 50 mM Tris-HCl, pH 9.0, at 25 °C for 30 min, and the digestions were stopped by acidification with 5% trifluoroacetic acid. Aliquots were applied to an Ultrasphere ODS reverse-phase column (Beckman Instruments; 4.6 mm × 25 cm, 5 μm). In this case, fractionation was performed using a Beckman System Gold program beginning at 0.008% trifluoroacetic acid and 80% acetonitrile, with peptide fragments being detected at 220 nm. Amino acid analysis of each fragment was performed as described previously.

**Vmax** and K0.5 values were measured using substrates at concentrations ranging from 10 to 50 μM with a final concentration of enzyme of 10 μM in 50 mM Tris-HCl, pH 9.0, at 25 °C. Aliquots were removed at various times, the digestions were stopped by acidification with 5% trifluoroacetic acid, and samples were applied to an Ultrasphere ODS reverse-phase column (Beckman Instruments; 4.6 mm × 25 cm, 5 μm). In this case, fractionation was performed using a Beckman System Gold program beginning at 0.008% trifluoroacetic acid and 80% acetonitrile followed by a 6%/min gradient to 0.032% trifluoroacetic acid and 32% acetonitrile. The gradient was held for 2.5 min, and then a 2.0%/min gradient to 0.04% trifluoroacetic acid and 40% acetonitrile was initiated. The peak height of the native substrate(s) was identified, and the decrease in peak area and height was used to determine the rate of peptide degradation. K0.5 and Vmax values were measured by using Hyperbolic Regression Analysis.2

**RESULTS**

**Enzyme Purification**—Utilizing simulated physiological conditions (10 mM Tris, pH 7.3, 150 mM NaCl), it was found that extraction of pollen at room temperature yielded large amounts of activity in less than 1 min, which peaked within 15 min (data not shown). However, enzyme stability allowed for a 24-h extraction at 4 °C in 10 mM ammonium bicarbonate, pH 8.0, in order to obtain the highest recovery of enzyme.

After centrifugation to remove remnants of the solubilized pollen grain, the supernatant was found to contain all activities

2 The Hyperbolic Regression Analysis program written by J. S. East-erby (University of Liverpool, United Kingdom) was obtained through shareware.
against N-Suc-Ala-Ala-Pro-Phe-pNA, with no measurable enzyme activity present in the reconstituted pellet. The use of a DEAE-Sephadex ion exchange chromatography step resulted in the removal of an appreciable amount of pigment and some contaminating protein, despite the fact that all enzyme activity passed unretarded through the column. The second step (Cibacron blue Sepharose CL-6B column chromatography) removed all of the remaining pigment as well as some contaminating proteins. Fortunately, the hydrophobic interaction column phenyl-Sepharose weakly bound the enzyme in 25% ammonium sulfate, while other proteins were tightly bound. Fig. 1 illustrates the importance of the hydrophobic interaction column, which removed much of the contaminating protein and helps visualize previously faint/absent bands. All other steps were required in order to obtain a purified preparation of enzyme and resulted in isolation of a single protein of mass 82 kDa, which corresponded with the increasingly prominent protein band observed during purification using Tricine SDS-PAGE, and was in agreement with the estimated mass obtained during gel filtration of the crude extract (data not shown). Significantly, this molecular mass is markedly different from any of the previously described major ragweed allergens (8–43 kDa) (26). Despite the fact that six columns were used, each was necessary, and attempts to change or eliminate a step adversely affected purification and decreased the yield. The isolation procedure developed here resulted in the purification of 614 μg of enzyme from 100 g, dry weight, of pollen, with a 9.0% yield and a 1900-fold purification (Table I).

SDS-PAGE Analysis—After reduction and boiling in SDS, the enzyme migrated as a single band at 82 kDa in agreement with that obtained by gel filtration (Fig. 1). Confirmation that this was indeed the desired proteinase was made by labeling with [35S]-DFP before SDS treatment, running the sample in a Tricine SDS-PAGE system, and exposing the gel to autoradiography (4-day exposure). This also resulted in the production of a band with an apparent mass of 82 kDa (Fig. 2).

NH₂-terminal Sequence Analysis—The NH₂-terminal sequence (NAASDIIGVMDTGI) was obtained by sequencing the band corresponding to the 82-kDa protein during SDS-PAGE. This was confirmed by also analyzing the radiolabeled band after blotting to a PVDF membrane. The sequence obtained had no homology with any other known proteases after comparison in GenBank™.

Stability—The enzyme was found to be stable over the pH range 4.5–10.0 for over 12 h at room temperature. It was frequently stored at pH 5.5 for 2–3 weeks at 4 °C and lost <2% of its activity after long term storage at −80 °C, both lyophilized and in solution.

Inhibition Profile—Incubation of the enzyme with DFP or TPCK resulted in total loss of activity, supporting its classification as a chymotrypsin-like serine proteinase. However, 3,4-dichloroisocoumarin was a poor inhibitor, and incubation with representatives of all other class-specific inhibitors resulted in no loss of enzyme activity (Table II). Known protein inhibitors, including avian ovomucoids, Kunitz-type trypsin inhibitors and the specific serpins α-1-PI, antithrombin III, and α-1-antichymotrypsin were also unable to inhibit the enzyme. Indeed, as is shown later, α-1-PI underwent limited proteolysis by this enzyme. A variety of synthetic inhibitors, including coumarins, peptidyl chloromethyl ketones, and organophosphates were also tested, several of which were effective in inhibiting the enzyme (Table III). The best of these contained Phe in the P₁ position and Pro in the P₂ position.

Protein and Peptide Degradation—The enzyme did not digest azocasein but did cleave small synthetic substrates at a pH optimum of 9.0. K_m and V_max values for the hydrolysis of several of these substrates are given in Table IV. The amido-lytic activity of the enzyme on synthetic peptide substrates agreed with the inhibitor studies, where the peptidase exhibited a preference for Phe in the P₁ position, with both Leu and Met also being suitable alternatives. However, Ala and Val were not efficient substitutes at the P₁ position, although in the P₂ position Val did increase hydrolysis rates. Pro was observed to be the best P₂ substituent, with there being no tolerance for Phe or Ala at that position. However, when the P₃ site was Phe, Val, or Leu, cleavage was also accelerated. Significantly, mono- and dipeptide substrates were not cleaved, while tripeptides were hydrolyzed only when Phe was present in both the P₁ and P₂ or P₃ positions.

Serpín Inactivation—The ragweed endopeptidase, which had shown no activity toward anything larger than peptide sub-

![Fig. 1. SDS-PAGE of fractions from the purification of serine endopeptidase from ragweed (A. artemisiifolia) pollen. Lanes 1 and 10, molecular mass markers (rabbit muscle phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; bovine erythrocyte carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa; bovine milk α-lactalbumin, 14.4 kDa). The following lanes contained boiled and reduced samples. Lane 2, extract of ragweed (A. artemisiifolia) pollen; lane 3, ammonium sulfate precipitate from extract; lane 4, DEAE-Sephadex ion-exchange; lane 5, Cibacron blue Sepharose CL-6B affinity column eluate; lane 6, phenyl-Sepharose CL-4B hydrophobic interaction column eluate; lane 7 Sepaherc S-200 Superfine column wash; lane 8, Mono S HR 5/5 FPLC column eluate; lane 9, purified ragweed pollen serine endopeptidase from TSK-GEL G3000SW gel filtration column wash.](http://www.jbc.org/)

### Table I

| Purification of ragweed pollen proteinase |
|-----------------------------------------|
| Fraction step | Total activity | Total protein | Specific activity | Purification Yield |
|---------------|---------------|---------------|------------------|-------------------|
|               | units mg      | units/mg      | %                |
| Crude extract | 129,000       | 13,700        | 9.4              | 1.0               | 100 |
| Ammonium sulfate (30–70%) | 82,400 | 4700 | 17.4 | 1.8 | 63 |
| DEAE-Sephadex | 79,100 | 1800 | 43.6 | 4.6 | 61 |
| Blue Sepharose | 61,200 | 412 | 148 | 15.7 | 47 |
| Phenyl-Sepharose | 43,200 | 104 | 415 | 44.1 | 33 |
| Sephadex S-200 | 28,600 | 3.9 | 7300 | 781 | 22 |
| Mono-S, FPLC | 24,500 | 1.8 | 13,000 | 1,450 | 18 |
| Gel filtration-TSK | 11,400 | 0.614 | 18,000 | 1,900 | 9 |

* Based on enzymatic activity using Suc-Ala-Ala-Pro-Phe-pNA, where 1 unit = 1 nmol of pNA released per min.

* 100 g of pollen extracted.
strates, was tested to see if it formed complexes with human plasma proteinase inhibitors. As mentioned earlier, no inhibition was found with any of the three serpins tested, and no complex formation could be detected after analysis by SDS-PAGE. Rather, the enzyme was found to inactivate α-1-PI, resulting in the cleavage of the inhibitor primarily at the P$_1$-P$_1'$ reactive site. This was determined by a slight decrease in the molecular mass of α-1-PI (Fig. 3), which was observed using SDS-PAGE and Western blotting with rabbit polyclonal antihuman α-1-PI, and visualized with enhanced chemiluminescence. Lane 1, native α-1-PI; lane 2, α-1-PI incubated with trypsin; lane 3, α-1-PI incubated with ragweed endopeptidase.

**Enzyme Specificity**—The ragweed peptidease cleaved both the insulin β-chain and the neuropeptides VIP and substance P quite efficiently, as shown in Table V. Hydrolysis occurred primarily after leucyl residues, despite the fact that phenylala-nine in the P$_1$ position appeared to be preferred with synthetic substrates and inhibitors. These results could be due to the presence of arginine residues near the putative leucine residue cleavage site, although this remains to be proven. For VIP, two major cleavage sites were present, while in the case of substance P a single peptide bond was hydrolyzed (Table V). The degradation of both neuropeptides was extremely efficient, occurring at low E:S molar ratios (1:5000). The kinetic parameters associated with their use as substrates for the ragweed

**TABLE II**

| Inhibitor | Class | Relative activity % |
|-----------|-------|---------------------|
| DFP       | Serine| 0                   |
| TPCK      | Cysteine/serine | 0         |
| TLCK      | Cysteine/serine trypsin-like | 100          |
| Leupeptin | Cysteine/serine | 100          |
| DCIC      | Serine | 87                   |
| E-64      | Cysteine | 100          |
| EDTA      | Metallo | 100          |
| Pepstatin | Aspartic | 100          |

**TABLE III**

| Inhibitor | IC$_{50}$ (mM) |
|-----------|---------------|
| Z-Phe-Ck | NI*            |
| Ac-Leu-Phe-Ck | NI       |
| Phe-Leu-Phe-Ck | 0.880  |
| Z-Gly-Leu-Phe-Ck | 0.024  |
| Z-Gly-Gly-Phe-Ck | NI     |
| Z-Gly-Gly-Ala-Ck | 0.450  |
| Ac-Ala-Ala-Ala-Ala-Ck | 0.360  |
| Ac-Ala-Ala-Pro-He-Ck | NI     |
| Ac-Ala-Ala-Phe-Ala-Ck | NI     |
| Ac-Ala-Leu-Ck | NI        |
| Z-Phe(P)-(OPh$_2$) | NI   |
| Cbz-Phe-Phe(P)-(OPh$_2$) | NI   |
| Cbz-Pro-Phe(P)-(OPh$_2$) | 1.000 |
| Cbz-Leu-Phe(P)-(OPh$_2$) | NI     |
| Cbz-Phe-Leu-Phe(P)-(OPh$_2$) | 0.0007 |
| Cbz-Pro-Phe-Phe(P)-(OPh$_2$) | 0.0006 |
| Cbz-Val(P)-(OPh$_2$) | NI     |
| Cbz-Val-Val(P)-(OPh$_2$) | 100.00 |
| Cbz-Val-Ala(P)-(OPh$_2$) | NI     |
| Cbz-Pro-Val-Phe(P)-(OPh$_2$) | 0.033  |
| Boc-Val-Pro-Val(P)-(OPh$_2$) | 0.005  |
| Boc-Ala-Pro-Val(P)-(OPh$_2$) | NI     |
| DFP      | 0.004         |

Results are for a 30-min incubation at 25 °C, final inhibitor concentration of 10 mM in 10% Me$_2$SO, 200 mM bis-Tris, pH 9.0. *NI, no inhibition.

**TABLE IV**

| Substrate | $V_{max}$ | $K_m$ | $V_{max}/K_m$ |
|-----------|-----------|-------|---------------|
| Bz-Arg-pNA | 0.01      |       |               |
| Suc-Leu-pNA | 0.01     |       |               |
| Suc-Ph-pNA  | 0.01      |       |               |
| Suc-Ala-Ala-Ala-pNA | 0.01 |       |               |
| Suc-Ala-Ala-Leu-pNA | 0.01 |       |               |
| Suc-Ala-Ala-Phe-pNA | 0.01     |       |               |
| Suc-Ala-Ala-Val-pNA  | 0.01     |       |               |
| Suc-Ala-Pho-Leu-pNA | 0.01     |       |               |
| Suc-Gln-Pro-Phe-pNA  | 0.01     |       |               |
| Suc-Gly-Gly-Leu-pNA | 0.01     |       |               |
| Suc-Leu-Pro-Phe-pNA  | 0.01     |       |               |
| Suc-Phe-Leu-Phe-pNA  | 0.6       | 0.13  | 65.8          |
| Suc-Phe-Pho-Leu-pNA  | 0.01     |       |               |
| Suc-Phe-Pro-Phe-pNA  | 10.961    | 1.46  | 7.5           |
| Suc-Phe-Pro-Leu-pNA  | 11.628    | 0.28  | 41.5          |
| Suc-Pro-Leu-pro-pNA  | 0.1       |       |               |
| Suc-Pro-Val-pro-pNA  | 0.1       |       |               |
| Suc-Ala-Ala-Leu-pNA  | 0.01     |       |               |
| Suc-Ala-Ala-Pro-Val-pNA | 0.1     |       |               |
| Suc-Ala-Ala-Pro-Ala-pNA | 0.1 |       |               |
| Suc-Ala-Ala-Pro-Leu-pNA | 77.9 | 1.68  | 46.4          |
| Suc-Ala-Ala-Pro-Met-pNA | 80.4 | 1.87  | 42.9          |
| Suc-Ala-Ala-Pro-Pro-pNA | 58.0 | 0.32  | 182.2         |
| Suc-Ala-Ala-Ala-Ala-pNA | 30.9 | 0.96  | 32.2          |
| Suc-Ala-Ala-Pro-Leu-pNA | 95.2 | 0.35  | 286.9         |
| Suc-Ala-Ala-Pro-Phe-pNA | 79.6 | 0.18  | 422.4         |
| Suc-Glu-Pro-Val-Pro-pNA | 5.0 | 0.65  | 7.7           |
| Suc-Leu-Pro-Val-Pro-pNA | 80.5 | 0.24  | 332.1         |
| Suc-Met-Val-Pro-Phe-pNA | 91.4 | 0.29  | 311.1         |
| Suc-Phe-Val-Pro-Phe-pNA | 99.0 | 0.24  | 411.1         |
| Suc-Phe-Ala-Ala-pNA | 0.01          |       |               |

**FIG. 3.** Western blot analysis of ragweed endopeptidase-modified α-1-proteinase inhibitor. The proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, reacted with rabbit polyclonal antihuman α-1-PI, and visualized with enhanced chemiluminescence. Lane 1, native α-1-PI; lane 2, α-1-PI incubated with trypsin; lane 3, α-1-PI incubated with ragweed endopeptidase.
proteinase are given in Table VI and indicate that in vivo their degradation could be of physiological significance relative to the maintenance of homeostasis within the lung (19, 27).

**DISCUSSION**

Species of *Ambrosia* are by far the most important cause of pollinosis in North America, with *A. artemisiifolia* (common/short ragweed) and *Ambrosia trifida* (giant ragweed) accounting for more cases of allergic rhinitis and its related diseases than all other plants combined (28). Initially, pollen grains from wind-pollinated (anemophilous) plants make contact with human tissues, absorb moisture, swell, and burst open, releasing the mobile proteins held within their walls in seconds. In the case of allergic rhinitis, this would occur when pollen is deposited on the mucous membranes of the upper respiratory tract during inhalation releasing up to 22 allergens as defined by reactivity against human IgE (29).

At present, five significant human allergens from common/short ragweed pollen, which cause a hypersensitivity reaction, have been purified to homogeneity and biochemically characterized. There has been some sequence homology with other proteins of widely differing functions, some possessing a variety of inhibitory as well as enzymatic activities (30). However, with the primary exception that each can stimulate the production of IgE-specific antibodies, which bind to receptors on mast cells and stimulate their degranulation, one can only speculate on other functions for these allergens based on their homologies with enzymes or inhibitors from other species (31).

A variety of readily leachable enzymes have been shown to be present within the pollen walls, making up a sizable proportion of the mobile proteins in the pollen grain (5, 32). While the biological function of these enzymes is believed to be connected with normal pollen function(s) during germination (33), no effort has been made to determine whether they could play a role in allergenic reactions.

In this report we have described the purification and properties of a novel endopeptidase from common/short ragweed pollen that could play a significant role in the pathobiological reactions that occur during pollinosis, despite the observation that it is not responsible for initiating hypersensitivity reactions. The enzyme is apparently a serine proteinase of chymotrypsin-like specificity, based on its inhibition by both DFP and TPCK (Table II), as well as its preference for Phe and Leu residues as demonstrated by peptidyl substrates and inhibitors (Tables III and IV). The kinetic data were obtained under normal laboratory conditions (25 °C), and the rates at physiological temperatures (37 °C) are expected to be higher. In parallel with a serine peptidase from mesquite pollen (34), the proteolytic activity of the ragweed enzyme in vitro seems to be limited to peptides because of its inability to degrade all macromolecular substrates tested. Such limitation is apparently due to a highly restricted specificity of the extended substrate binding site, involving at least four amino acid residues preceding the cleaved peptide bonds.

It is likely that the biological function of the ragweed peptidase involves participation in germination of pollen and/or pollination, through the specific cleavage of a plant precursor protein(s). Indeed, it does possess some endopeptidase activity as documented by an ability to inactivate human α-1-PI through cleavage at the P1-P1′ Met-Ser reactive site peptide bond of this neutrophil elastase-specific inhibitor. However, it should be pointed out that the reactive site loop of this and several other members of the serpin family is easily exposed for binding to target proteinases and, therefore, more readily accessible to endopeptidase cleavage (35). From a pathophysiological point of view, such an inactivation may be highly significant, since it would markedly alter the proteinase-proteinase inhibitor balance at sites where pollen is deposited, allowing the possibility of unregulated tissue proteolysis by elastase released from neutrophils during phagocytosis of pollen particles.

In the respiratory tract, neuropeptides that are located to neurons, neuroendocrine cells, and inflammatory cells are believed to be responsible for the regulation of airway secretion, vascular permeability, and bronchomotor tone (27). VIP and substance P are proposed to be essential, serving as neurotransmitters modulating airway caliber, and it is their widespread distribution and numerous physiological effects that make their uncontrolled inactivation excellent candidates for important roles in asthma (36). The disruption of their regulatory functions would clearly cause air flow limitation and an increase in bronchial hyperactivity, two pathophysiological effects that occur during the development of asthma. While neuropeptide regulation is believed to depend on the action of inactivating enzyme(s) that are responsible for their specific and rapid degradation, little is known as to how such peptidases are controlled, and there have been many reports of different peptidases that may be involved in neuropeptide degradation (37, 38).

Airway exposure to ragweed pollen that results in antibody-mediated hypersensitivity reactions, are known to be prone to more specific hyperresponsiveness (39, 40). This increased airway hyperresponsiveness to a variety of stimuli is a major characteristic of asthma. It has been found that in chronic allergic inflammation, lungs were more sensitive to the contractile effects of substance P and less sensitive to the relaxant effects of VIP (41). This increased responsiveness to contraction and diminished responsiveness to relaxation could be conditions further compromised by the hydrolyzing effects of the ragweed endopeptidase described here, which provides in vitro evidence that this enzyme rapidly converted VIP and substance P into fragments that are no longer able to perform their in vivo functions (42). There are few published data for the estimation of the concentrations of these peptides in the actual tissue microenvironment where they are released, and much higher concentrations are certain to be attained locally than are predicted by assays of peptide content in tissue homogenates (27). Therefore, it is reasonable to suggest that the observed rates of cleavage of VIP and substance P by pollen proteinases may be

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**Table V**

| Peptide | Cleavage specificity by ragweed pollen proteinase |
|---------|--------------------------------------------------|
| VIP     | His-Ser-Asp-Ala-Val-Phe-↓-Thr-Asp-Asp-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu↓-Aas-Ser-Ile-Leu-Aas-NH₂ |
| Insulin β-chain | Phe-Val-Asp-Glu-His-Leu↓-Cys-Gly-Ser-His-Leu↓-Val-Glu-Ala-Leu↓-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala |
| Substance P | Arg-Pro-Lys-Pro-Gln-Glu-Phe-↓-Phe-Gly-Leu-Met |

**Table VI**

| Peptidase | Kinetic constants for the hydrolysis of neuropeptides by ragweed pollen proteinase |
|-----------|----------------------------------------------------------------------------------|
|          | *V*ₘₐₓ | *K*ₘ | *V*ₘₐₓ/*K*ₘ |
| Vasoactive intestinal peptide | 1769.9 | 34.5 | 51.2 |
| Substance P | 1636.8 | 52.7 | 31.0 |
biologically significant. We believe that the ability of the ragweed enzyme to degrade these neuropeptides, in conjunction with other proteins present in the pollen grain, may be important in allergy-related asthma and general lung dysfunction due to the altered lung physiology after pollen exposure.

We hypothesize that during the exposure to ragweed pollen the degradation of VIP and substance P by the pollen endopeptidase described here, as well as by other peptidases present in pollen walls, could cause a disruption in the balance between contractile and relaxant effects of neuropeptides in the lungs and, therefore, be a major factor in the development of pollen allergy-related asthma. Indeed, the ability of pollen-derived endopeptidases to be solubilized within the upper airways and interrupt the regulation of lung airway function by degradation of small vasoactive peptides may have significant importance during the development of allergic and/or asthmatic reactions after pollen exposure.

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Purification and Characterization of a Novel Endopeptidase in Ragweed (Ambrosia artemisiifolia) Pollen

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