



RAGWEED POLLEN PROTEOLYTIC ENZYMES: POSSIBLE ROLES IN ALLERGIES AND ASTHMA

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Key Word Index—Allergic rhinitis; α -1-proteinase inhibitor (α -1-PI); *Ambrosia artemisiifolia*; compositae; chymotrypsin; hay fever; neuropeptide; proteolytic enzyme; ragweed; serine; trypsin.

Abstract—Extracts from ragweed pollen grains contain novel trypsin and chymotrypsin-like serine peptidases which are described in this report. The molecular mass of the chymotrypsin-like enzyme was 82 kDa, had a pH optimum near 9.0, and its activity was unaffected by chelating or reducing agents. It was inhibited by diisopropyl fluorophosphate (DFP), a general serine class inhibitor, and more specifically *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), a chymotrypsin-like proteinase inhibitor. In addition to various synthetic substrates, the neuropeptides, vasoactive intestinal peptide (VIP) and substance P, which are required for normalized lung functions, were also rapidly hydrolysed. Activity toward protein substrates was not detected with the exception of the inactivation of α -1-proteinase inhibitor (α -1-PI) which occurred through cleavage within the reactive site loop.

The 'trypsin-like' enzyme has a molecular mass near 80 kDa, a blocked N-terminus, a pH optimum near 9.0, and requires Ca^{++} for stability and activity, but not reducing agents. It is inhibited by DFP, and more specifically the trypsin-like proteinase inhibitor, *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK). Again, activity toward protein substrates was not detected, but various synthetic substrates and biologically active peptides were efficiently cleaved. Significantly, atrial natriuretic peptide (ANP) and angiotensin 2 (ATII), whose degradation would amplify kinin activity and influence inflammatory diseases of the respiratory tract and nasal passages, were also rapidly hydrolyzed. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Airborne pollen was recognized more than a century and a half ago as a leading source of morbidity among atopic subjects. Hay fever allergies, generically referred to as seasonal allergic rhinitis or pollinosis, have emerged from a medical curiosity to one of the most widespread and clinically important of all the allergic diseases [1]. Pollen grains from virtually all seed plants, from trees and grasses in the spring and summer and weeds in summer and fall, are responsible for the widespread hay fever (allergic rhinitis or pollinosis) outbreaks.

Perennial allergic rhinitis is a year-round allergy of people who are sensitive, usually to household allergens, with no links to changing seasons. These irritants are usually dust, dust mites, molds, feathers, animal dander or insect excreta [2]. Seasonal allergic rhinitis is the most common atopic disorder, a condition which appears during a defined season when aeroallergens

are abundant in outdoor air. Common diseases associated with allergic rhinitis are hay fever, allergic asthma and eczema [3]. Nearly 30 million Americans suffer from the symptoms of hay fever resulting with severe economic and physical repercussions. These individuals annually spend approximately 225 million dollars on physician services, 300 million dollars on prescription drugs and nearly 2 billion dollars on over-the-counter allergy medications [4].

The Heliantheae (sunflower) family contains the subtribe *ambrosia*, whose members are mostly wind-pollinated species that provoke the most severe allergic reactions of all known plants. Species of *Ambrosia* are the major cause of pollinosis in North America, with *Ambrosia artemisiifolia* (common/short ragweed) being responsible for more cases of allergic rhinitis and its related diseases than all other plants combined. As a result, ragweed pollen is responsible for the majority of late summer hay fever [5]. The extremely small particle size of ragweed allows this pollen to be carried hundreds of kilometers and reports of ragweed hay fever are common in Central and South America, Europe, the Mediterranean, and

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clinical symptoms are now being reported as far east as Asia. These increasingly varied environments and climates allow this hearty plant opportunities to pollinate at different times of the year, often for extended periods, causing increased human exposure and resulting in the sensitization of more of the population [6–9].

The complex mixture of proteins that are released from ragweed pollen grains have been shown to be some of the most powerful antigens/allergens known. Indeed, of the 52 antigens present in an aqueous extract of ragweed pollen, at least 22 are allergens as defined by reactivity with human IgE [10]. Six major allergens make up to 12% of extracted protein. However, none have been found to have any biological activity other than activation of mast cells [11, 12].

To date, research has shown that the major allergens from ragweed pollen cause only mast cell degranulation and possess no enzymatic activity. This is in contrast to some of the major allergens from other organisms which contained proteolytic activity that either caused or antagonized the type I hypersensitivity response [13–15]. Thus, it was clear that the involvement of proteolytic enzymes in pollen allergies had been ignored. For this reason, experiments were begun to determine if proteolytic enzymes were present in ragweed pollen, and whether they might add to the complications already known to accompany allergic rhinitis and its associated diseases.

RESULTS AND DISCUSSION

Characteristics of ragweed proteolytic enzymes

Preliminary experiments revealed that multiple proteolytic factors were present in ragweed pollen extracts that hydrolyzed various synthetic substrates. Two of the activities which were detected were towards cleavage of the synthetic substrates Suc-Ala-Ala-Pro-Phe-pNa and Bz-Arg-pNa. Initial purification steps enabled separation of the activities, clearly indicating two distinct enzymes, and the detailed properties of each of these novel proteolytic enzymes has been recently described [16, 17].

It should be noted that initial experiments on the ability of ragweed extracts to hydrolyse protein substrates (e.g. casein/azocasein) resulted in no detectable proteolytic activity. However, the presence of a peptidase activity was suspected by testing on synthetic peptide substrates, suggesting that both enzymes should be classified as peptidases, rather than proteinases. The effect of various inhibitors allowed the classification of both proteins as serine peptidases, since DFP totally inhibited both enzymes (Table 1), while representatives of all other class specific inhibitors were not effective. This was confirmed by both DFP-radiolabelling and synthetic substrate studies.

Properties of the chymotrypsin-related peptidase

The first enzyme studied has been classified as a serine peptidase with chymotrypsin-like activity. This

was based on its inhibition by DFP, a general serine-class proteinase inhibitor, and *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), a more specific inhibitor of chymotrypsin-related proteinases (Table 1). These conclusions were further supported by studies with synthetic peptide substrates and inhibitors, where a preference for Phe and Leu residues in the P₁ position was noted [Table 2(A) and Table 3(A)], as well as for Pro in the P₂ position. The enzyme had a molecular mass near 82 kDa according to gel filtration and SDS-polyacrylamide gel electrophoresis, a pH optimum near 9.0, and was unaffected by chelating agents. Amino terminal sequence analysis through 17 residues gave a structure which showed no similarity to other proteins. Significantly, no degradation of proteins or peptides less than three amino acids in length could be observed, strongly supporting the classification of this enzyme as being a strict serine-class endopeptidase.

Properties of the trypsin-related peptidase

This enzyme has been characterized as a serine peptidase with trypsin-like specificity, again based on inhibition by DFP and strict susceptibility to inhibition by *N*-*p*-tosyl-L-Lysine chloromethyl ketone (TLCK) (Table 1), a specific inactivator of trypsin-related proteinases. Synthetic substrates were efficiently cleaved with a strong preference for Arg in the P₁ position and either Arg or Gly ion P₂. This specificity was further confirmed through studies with specific chloromethyl ketone or phosphonate inhibitors [Table 3(B)]. The enzyme has a molecular mass near 80 kDa, a pH optimum near 9.0, and requires Ca⁺⁺ for stability and activity. No amino terminus could be detected suggesting that this site was blocked. Again, the enzyme could not degrade proteins; however, it could hydrolyse single and di-amino substrate *p*-nitroanilides [Table 2(B)]. Thus, it is also classified as a serine-type endopeptidase.

Enzymatic hydrolysis of regulatory neuropeptides and protein by ragweed endopeptidases

The original study was initiated in an attempt to isolate and characterize pollen proteolytic enzymes which might specifically degrade proteins or peptides involved in normal respiratory function. For this reason both serine endopeptidases from short ragweed (*Ambrosia artemisiifolia*) pollen were examined, *in vitro*, to determine if either could efficiently degrade essential neuropeptides. The 'chymotrypsin-like' serine peptidase was shown to be able to hydrolyse two neuropeptides, substance P and vasoactive intestinal peptide, each of which is essential for regulating airway response [Table 4(A)] [18–21]. In addition, it was also found that this enzyme could inactivate α -1-PI, the primary regulating inhibitor of human neutrophil elastase. This action alone could implicate the enzyme in respiratory distress since its inactivation would

Table 1. Effect of Class Specific Inhibitors on the Amidolytic Activity of Ragweed Pollen Peptidases

Inhibitor	Class	Chymotrypsin-like peptidase % Relative Activity	Trypsin-like peptidase % Relative Activity
DFP	Serine	0	0
TPCK	Cysteine/Serine	0	100
TLCK	Cysteine/Serine	100	0
Leupeptin	Cysteine/Serine	100	100
DCIC	Serine	87	100
E-64	Cysteine	100	100
EDTA	Metallo	100	100
Pepstatin	Aspartic	100	100

Results are for a 30 min incubation at 25°, final inhibitor concentration of 10 mM in 15% DMSO, 200 mM Bis-Tris pH 9.0.

Table 2A. Amidolytic activity of ragweed pollen proteinase

Substrate	V_{max} mM min ⁻¹	K_m mM	V_{max}/K_m
Bz-Arg-pNA	<0.1		
Suc-Leu-pNA	<0.1		
Suc-Phe-pNA	<0.1		
Suc-Ala-Ala-Ala-pNA	<0.1		
Suc-Ala-Ala-Leu-pNA	<0.1		
Suc-Ala-Ala-Phe-pNA	<0.1		
Suc-Ala-Ala-Val-pNA	<0.1		
Suc-Ala-Phe-Leu-pNA	<0.1		
Suc-Ala-Pro-Leu-pNA	<0.1		
Suc-Gln-Pro-Phe-pNA	<0.1		
Suc-Gly-Gly-Leu-pNA	<0.1		
Suc-Leu-Pro-Phe-pNA	<0.1		
Suc-Phe-Leu-Phe-pNA	8.6	0.13	65.8
Suc-Phe-Phe-Leu-pNA	<0.1		
Suc-Phe-Pro-Phe-pNA	10.9	1.46	7.5
Suc-Phe-Val-Phe-pNA	11.6	0.28	41.5
Suc-Pro-Phe-Leu-pNA	<0.1		
Suc-Val-Pro-Leu-pNA	<0.1		
Suc-Val-Pro-Phe-pNA	<0.1		
Suc-Ala-Ala-Phe-Leu-pNA	<0.1		
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-Phe-pNA	58.0	0.32	182.2
Suc-Ala-Ala-Val-Ala-pNA	30.9	0.96	32.2
Suc-Ala-Leu-Pro-Phe-pNA	95.2	0.35	269.4
Suc-Ala-Phe-Pro-Phe-pNA	79.6	0.18	422.4
Suc-Glu-Val-Pro-Phe-pNA	5.0	0.65	7.7
Suc-Leu-Val-Pro-Phe-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-Phe-pNA	<0.1		

The assay was performed at 25° in 200 mM Bis-Tris pH 9.0.

allow free elastase activity to degrade inflamed tissues and add to the complications of allergies and asthma [22, 23].

Table 2B. Amidolytic activity of ragweed pollen peptidase

Substrate	V_{max} nM/min	K_m mM	V_{max}/K_m
Bz-Arg-pNA	28.2	0.254	110.8
Z-Arg-pNA	16.4	0.055	298.7
Z-Arg-Arg-pNA	11.7	0.036	320.6
Z-Gly-Arg-pNA	73.8	0.255	289.0
Z-Lys-Arg-pNA	27.3	0.198	137.9
Z-Phe-Arg-pNA	7.9	0.127	62.3
Z-Trp-Arg-pNA	6.3	0.113	56.1
Z-Ala-Gly-Arg-pNA	47.2	0.178	265.4
Z-Arg-Gly-Arg-pNA	32.7	0.108	302.8
Mes-Phe-Gly-Arg-pNA	33.1	0.112	294.7
Z-Pro-Gly-Arg-pNA	19.3	0.070	274.6
Z-Val-Leu-Gly-Arg-pNA	6.1	0.052	117.4
Z-Tyr-Lys-Arg-pNA	21.4	0.207	103.4
Z-Leu-Ala-Arg-pNA	20.7	0.160	129.3
Z-Leu-Thr-Arg-pNA	30.1	0.123	245.2
Z-Glu-Phe-Arg-pNA	6.6	0.144	45.6
Z-Ser-Phe-Arg-pNA	4.4	0.193	23.0
Z-Lys-Phe-Arg-pNA	7.9	0.121	65.4
Z-Pro-Phe-Arg-pNA	4.3	0.159	27.1
Tos-Gly-Pro-Arg-pNA	27.6	0.273	101.1
Z-Ile-Pro-Arg-pNA	39.5	0.443	89.2
Z-Sar-Pro-Arg-pNA	40.5	0.283	143.0
Z-Phe-PIP-Arg-pNA	2.3	0.173	13.3
Z-Phe-Val-Arg-pNA	4.7	0.189	24.9

The assay was performed at 25° in 200 mM Bis-Tris pH 9.0.

We believe that the 'trypsin-like' serine peptidase may add to the hypersensitivity reaction, not by causing an imbalance in lung neuropeptides, but in altering the mediators of the renin-angiotension II/kallikrein-kinin systems [Table 4(B)]. These neuropeptides, which are highly susceptible to proteolytic inactivation, include atrial natriuretic peptide, a profound inhibitor of vascular contraction [24], and angiotensin II, a potent vasoconstrictor [25]. Disruption of these systems causes an increase in kinin flow resulting in

Table 3A. Effect of peptide inhibitors on the amidolytic activity of ragweed pollen proteinase

Z-Phe-CK	N.I.
Ac-leu-Phe-CK	N.I.
Phe-Leu-Phe-CK	0.880
Z-Gly-Leu-Phe-CK	0.024
Z-Gly-Gly-Phe-CK	N.I.
Z-Gly-Leu-Ala-CK	0.450
Ac-Ala-Ala-Ala-Ala-CK	N.I.
Ac-Ala-Ala-Pro-Ala-CK	0.360
Ac-Ala-Ala-Pro-Ile-CK	N.I.
Ac-Ala-Ala-Phe-Ala-CK	N.I.
Ac-Ala-Leu-CK	N.I.
Z-Phe ^P -(OPh ₂)	N.I.
Cbz-Phe-Phe ^P -(OPh ₂)	N.I.
Cbz-Pro-Phe ^P -(OPh ₂)	1.000
Cbz-Leu-Phe ^P -(OPh ₂)	N.I.
Cbz-Phe-Leu-Phe ^P -(OPh ₂)	0.0007
Suc-Val-Pro-Phe ^P -(OPh ₂)	0.0006
Cbz-Val ^P -(OPh ₂)	N.I.
Cbz-Val-Val ^P -(OPh ₂)	> 100.00
Cbz-Ala-Val ^P -(OPh ₂)	N.I.
Cbz-Pro-Val ^P -(OPh ₂)	0.033
Boc-Val-Pro-Val ^P -(OPh ₂)	0.005
Boc-Ala-Pro-Val ^P -(OPh ₂)	N.I.
DFP	0.004

Results are for a 30 min incubation at 25°, final inhibitor concentration of 10 mM in 10% DMSO, 200 mM Bis-Tris pH 9.0.

NI, No Inhibition.

Table 3B. Effect of peptide inhibitors on the amidolytic activity of ragweed pollen peptidase

Inhibitor	IC ₅₀ (mM)
Z-Arg-CK	0.490
Z-Trp-Arg-CK	1.460
Z-Glu-CK	NI
Z-Ala-Gly-Arg-CK	0.430
Z-Pro-Gly-Arg-CK	0.370
Z-Glu-Phe-Arg-CK	1.570
Z-Lys-Phe-Arg-CK	3.320
Z-Phe-Phe-Arg-CK	1.100
Z-Pro-Phe-Arg-CK	1.350
Z-Ser-Phe-Arg-CK	2.120
Z-Gly-(OPh ₂)	NI
Z-Lys-(OPh ₂)	0.510
Boc-Phe-Leu-Gly-(OPh ₂)	0.260
DCIC	0.560
DFP	0.130

Results are for a 30 min incubation at 25°, final inhibitor concentration of 10 mM in 10% DMSO, 200 mM Bis-Tris pH 9.0.

NI, No Inhibition.

vascular permeability, plasma leakage and oedema. In the upper respiratory system this results in inflammation and congestion [26]. In the lower respiratory tract, this same excess of kinins is known to augment asthmatic complications, which are also present after ragweed pollen exposure [27, 28].

EXPERIMENTAL

Protein extraction, enzyme purification and kinetics.

Pollen extracts were prepared and enzymes isolated as previously described [16, 17]. For specificity studies, substrates were incubated at an enzyme:substrate molar ratio of 1:1000 in 50 mM Tris-HCl, 5 mM CaCl₂, pH 9.0, at 25° for 30 min with the digestions stopped by acidification with 5% trifluoroacetic acid. Bioactive peptide degradation was measured using an enzyme:substrate ratio of 1:5000 in 50 mM Tris-HCl, 5 mM CaCl₂, pH 9.0 at 25°. Aliquots were removed at various time periods and digestion was stopped by acidification with 5% trifluoroacetic acid. Peptide fragments were separated using HPLC, as described [16], with cleavage determined through amino acid analysis. *K_m* and *V_{max}* values were measured using substrates at concentrations ranging from 10 to 50 µM with a final concentration of enzyme of 10 nM in 50 mM Tris-HCl, pH 9.0 at 25°, and calculated using Hyperbolic Regression Analysis.*

SUMMARY AND CONCLUSIONS

Type I or immediate hypersensitivity occurs when an adaptive-immune response is directed against benign environmental antigens, such as pollen. The allergic reaction occurs instantly following contact with the antigen (allergen), resulting in the release of pharmacological mediators by IgE sensitized mast cells, and producing an acute inflammatory reaction with symptoms such as asthma or rhinitis [4]. It is only in recent years that 'allergy' has become synonymous with Type I hypersensitivity. Although it is well documented that the ragweed major allergens initiate hypersensitivity responses, it is necessary to study other factors present in pollen which may contribute to the complications associated with seasonal ragweed pollen exposure. This knowledge could assist in the development of additional treatments or preventive measures. Thus, this research was initiated to isolate a factor(s) not previously identified that could contribute to the complex set of events and take place during Type I immediate hypersensitivity reactions.

Ragweed pollen enzymes

Initially, pollen making contact with human tissue would react in the same way as one on the stigma. After absorbing moisture, the pollen grain would burst open and release the mobile proteins held in the walls within seconds. In the case of allergic rhinitis, this would occur by pollen being deposited on the mucous membranes. Upon contact, mucous liquid would solubilize the pollen and proteins would be released rapidly from the surface micropores and

* Hyperbolic Regression Analysis program written by J. S. Easterby (University of Liverpool, England) was obtained through shareware.

Table 4A. Cleavage specificity of peptides of ragweed pollen proteinase

VIP	His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Arg-Lys-Gln-Met-
	↓
	Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH ₂
Insulin Beta-chain	Phe-Val-Asn-Gln-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-Gln-Phe-Phe-Gly-Leu-Met
	↓
	↓ Signifies cleavage sites.

Table 4B. Cleavage specificity of peptides by ragweed pollen peptidase

Atrial Natriuretic Peptide	Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-
	↓
	Met-Asn-Arg-Ile-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg
Angiotensin 2	Asn-Arg-Val-Tyr-Ile-His-Pro-Phe
	↓
Angiotensin 1	Asn-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
VIP	His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-
	↓
	Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH ₂
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met
	↓
	↓ Signifies cleavage sites.

through apertures, where they could readily penetrate the mucous tissues.

A variety of enzymatic activities have been previously identified in ragweed pollen. Indeed, the pollen wall is known to contain several enzymes which are readily leachable and which make up a sizeable portion of the mobile protein in the pollen grain [29]. Certain enzymes escape in the first few minutes, while some are delayed for up to 10 min [30, 31]. In the case of the endopeptidases investigated here, release is essentially instantaneous.

Regulatory neuropeptides

A mechanism linking chronic airway inflammation to deranged physiological function is altered enzymatic inactivation of lung neuropeptides. Several peptides have been shown to serve as neurotransmitters modulating airway caliber, vascular tone, mucous secretion, and vascular permeability. Those regulatory peptides discussed here (substance P, VIP, AT II, and ANP) are initially synthesized as larger precursors that undergo proteolytic digestion to smaller, biologically active peptides within the cell of origin [32]. Such mature peptides may encounter a number

of enzymes that can either inactivate them or alter them to other specific biologically active forms. This delicate balance, achieved by expression of pre-pro forms and production of modifying/degrading enzymes, would be exceptionally susceptible to dysregulation by non-host peptidases which are not controlled by host inhibitors.

In this review, two novel serine endopeptidases have been described which possess the ability to exacerbate the complications of allergic rhinitis and pollen-induced asthma. It is believed that the biological function of these enzymes, and others present in pollen, are likely to be involved in fertilization and/or germination of the plant. In human disease, however, the chymotrypsin-like enzyme may play a role in pollen-induced asthma by hydrolysis of regulatory lung neuropeptides, and as a result, bronchoconstriction. In addition, it has the ability to inactivate the regulatory inhibitor α -1-PI, which would enable unregulated host-enzymes to degrade tissue. The trypsin-like enzyme may dysregulate the balance between the renin-angiotensin II and kallikrein-kinin systems, and would contribute to allergic rhinitis in the upper respiratory tract, and allergic asthma in the lower respiratory tract.

With no apparent natural inhibitors, these novel enzymes may be involved in both the degradation of regulatory neuropeptides and the inactivation of protective proteinase inhibitors during pollen-initiated allergic reactions. Much in the same way dust mite, fungal, and bacterial proteases are known to be involved in disease, it is suggested that the ragweed proteolytic enzymes described may play a crucial role in the diseases associated with pollen exposure.

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