

Isolation and Properties of an Angiotensin II-cleaving Peptidase from Mesquite Pollen

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Biochemical studies of pollen proteins have been focused, primarily, in investigating their roles as allergens. These molecules, some of which have enzymatic activity, act as antigens and initiate the production of IgE antibodies, leading to allergic and/or asthmatic responses. Included in this mixture of proteins are proteinases which, although they may or may not be allergenic, could still be involved in airway dysfunction. We have isolated an arginine-specific endopeptidase to homogeneity from mesquite (*Prosopis velutina*) pollen, a known wind-borne allergen, which has a molecular mass near 84 kDa by NaDodSO₄-gel electrophoresis, a pH optimum in the neutral to alkaline range, and a requirement for Ca²⁺ for stabilization. The enzyme is inhibited by diisopropyl fluorophosphate (DFP) and N-p-tosyl-L-lysine chloromethylketone but not by N-p-tosyl-L-phenylalanine chloromethylketone, EDTA, or iodoacetamide. It was also not inhibited by human plasma proteinase inhibitors nor several other naturally occurring plant and animal inhibitors. Cleavage by the endopeptidase was primarily on the carboxy-terminal side of arginine residues in peptides, whereas proteins such as kallikrein and prothrombin were only activated and/or degraded extremely slowly. Several bioactive peptides that may be involved in maintaining normal lung function were readily fragmented, including angiotensin II, a vasoconstrictor, and atrial natriuretic peptide, a modulator of vascular permeability, both of which were rapidly cleaved at low enzyme:substrate molar ratios. Thus, the pollen endopeptidase could be involved in exacerbating the development of asthma by inactivating bioactive peptides that have ameliorating effects in maintaining lung airway homeostasis.

Asthma is a chronic inflammatory disease, specifically affecting the airways of the lung. Its cause, while not well understood, occurs primarily after prolonged exposure to a number of irritants, including pollutants, dust, and pollen. At this time, however, very little is known with regard to the biochemical mechanisms that initiate either allergenic or asthmatic reactions.

Pollen is considered to be one of the major triggering agents of allergic diseases, resulting in (a) the production of immunoglobulins (IgE), (b) the degranulation of mast cells with the release of both histamine and chemoattractants, (c) the invasion of a variety of inflammatory cells into the lung, and (d) the release of hydrolytic enzymes, inflammatory mediators, and neuropeptides that cause inflammation and/or affect airway function (1). However, nothing is known at

the biochemical level with regard to the initial events that activate these processes. In particular, little attention has been paid to the fact that many of the major allergenic components in various pollens are hydrolytic enzymes (2, 3) which could, besides being allergens, actually take part in specific processes during the initiation of the allergenic reaction. Recently, however, it was shown that a cysteine proteinase from dust mites, referred to as Der I (4), was the major source of house dust allergens, being responsible for up to 80% of the anti-IgE responsive antibodies in allergic sera (5, 6). Furthermore, it was found that this enzyme could activate the kallikrein-kinin system in human plasma (7-9) and, therefore, exacerbate the inflammatory reactions associated with allergenic and asthmatic responses.

Our interest stems from the fact that many of the biochemical reactions that occur during the allergenic response must involve proteolytic enzymes. Therefore, the presence of exogenous, biologically active factors in pollen with activities similar to the Der I proteinase could augment or accelerate this reaction(s). In this report, we describe an endopeptidase that we have isolated from mesquite (*Prosopis velutina*) pollen, a major hayfever-associated allergen in southcentral and southwestern regions of the United States, whose properties suggest that it could take part in the inflammatory processes associated with both allergenic and asthmatic responses.

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Abbreviations: 4-(2-aminoethyl) benzene sulfonyl fluoride, AEBF; atrial natriuretic peptide, ANP; diisopropyl fluorophosphate, DFP; high pressure liquid chromatography, HPLC; N-p-tosyl-L-lysine chloromethyl ketone, TLCK; N-p-tosyl-L-phenylalanine chloromethyl ketone, TPCK; vasoactive intestinal peptide, VIP.

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Materials and Methods

Experimental Procedures

Bz-DL-Arg-pNA, Suc-Ala-Ala-Ala-pNA, Suc-Ala-Ala-Pro-Phe-pNA, MeO-Suc-Arg-Pro-Tyr-pNA, phenylmethylsulfonyl fluoride (PMSF), iodoacetamide, benzamidine, N-p-tosyl-L-lysine chloromethyl ketone (TLCK), N-p-tosyl-L-phenylalanine chloromethyl ketone (TPCK), antipain, soybean trypsin inhibitor, lima bean trypsin inhibitor, aprotinin, angiotensin II, substance P, neurotensin, atrial natriuretic peptide (ANP), dynorphin fragment 1-13, and vasoactive intestinal peptide (VIP) were from Sigma (St. Louis, MO). Diisopropyl fluorophosphate (DFP) and leupeptin were obtained from Calbio-chem (La Jolla, CA), ovomucoid from Worthington (Freehold, NJ), and 3,4-dichloroisocoumarin and 4-(2-aminoethyl) benzene sulfonyl fluoride (AEBSF) from Boehringer (Indianapolis, IN). S-2266 (D-Val-Leu-Arg-pNA), S-2302 (D-Pro-Phe-Arg-pNA), S-2251 (D-Val-Leu-Lys-pNA), S-2222 (Bz-Ile-Glu-(γ -OR)-Gly-Arg-pNA), S-2238 (D-Phe-Pip-Arg-pNA), and S-2484 (Glu-Pro-Val-pNA) were from Kavi-Vitrum. D-Phe-Pro-Arg-chloromethyl ketone was provided by Dr. James Powers, Georgia Institute of Technology, Atlanta, GA. Tomato leaf inhibitors I and II, potato leaf inhibitors PC 1 and 3, and tobacco leaf inhibitor T 1 were gifts from Dr. C. A. Ryan, Washington State University, Pullman, WA. Mesquite pollen was obtained from the Carl Hayden Bee Research Center, Tucson, AZ.

Methods

Enzyme purification. In initial experiments, it was found that either 0.1 M Tris-HCl, pH 8.0, 0.15 M NaCl, 5 mM CaCl₂ or 0.02 M Bis-Tris, pH 6.5, 5 mM CaCl₂ could be used to extract Bz-Arg-pNA activity from pollen, most activity being released within 7 h. However, after stirring for 48 h, maximal activity was released with Bis-Tris buffer (buffer A), and this was used throughout the purification scheme.

Mesquite pollen (100 g) was extracted in 400 ml of buffer A at 4° for 48 h, followed by centrifugation at 48,000 $\times$ g for 30 min. The supernatant was fractionated between 0.30 and 0.60 saturation with solid ammonium sulfate, the protein pellet being retained after centrifugation for 20 min at 48,000 $\times$ g. The precipitate was dissolved in buffer A (100 ml) and dialyzed against the same buffer for 4 h with two changes. The dialysate was adjusted to pH 4.5 with 0.4 M sodium acetate buffer, pH 3.8, centrifuged for 15 min at 48,000 $\times$ g, and readjusted to pH 6.5 with 1.0 M Tris-HCl, pH 8.0. It was then dialyzed overnight against buffer A with three changes.

The dialyzed solution was applied to a column of Cibacron blue Sepharose (4.0 $\times$ 40.0 cm), equilibrated with buffer A, and the column washed with 700 ml of the same buffer. All Bz-Arg-pNA activity passed unretarded through the column, was collected in 8.0-ml fractions, and pooled. This material was next applied to a column of DEAE-Sephacel (3.0 $\times$ 21.0 cm), equilibrated with buffer A, and washed with 500 ml of the same buffer. Activity was eluted with a linear gradient (1.0 liter total volume) from 0 to 0.5 M NaCl in buffer A, collected in 8.0-ml fractions and pooled.

The eluate from the DEAE-Sephacel column was applied to a column of phenyl-Sepharose (2.0 $\times$ 16.0 cm), equil-

ibrated with buffer A containing 2.0 M NaCl, and washed with 300 ml of the same buffer. Enzyme was eluted with a linear gradient (1.0 liter total volume) from 2.0 to 0 M NaCl in buffer A, collected in 8.0 ml fractions, and pooled. This solution was concentrated to 8.0 ml, using an Amicon PM30 membrane, applied to a column of Sephacryl S-100 (2.6 $\times$ 78.5 cm), equilibrated with buffer A containing 0.2 M NaCl and 5 mM CaCl₂. The column was developed with the same buffer system and enzyme collected in 4.0 ml fractions and pooled. This solution was then dialyzed overnight against 0.2 M Bis-Tris, pH 7.0, 5 mM CaCl₂ (buffer B) with two changes of buffer and concentrated to 8.0 ml.

The final step of purification involved the application of the dialyzed and concentrated Sephacryl S-100 active fraction to a Mono Q FPLC column (Pharmacia LKB Biotechnology, Inc.), equilibrated with buffer B. The column was washed with starting buffer for 10 min, followed by a 0 to 0.1 M NaCl gradient for 10 min, then a 0.1 to 0.25 M NaCl gradient for 45 min during which enzyme activity was eluted.

Molecular Weight Determination

The molecular weight of the purified enzyme was determined by both NaDodSO₄ gel electrophoresis (10), using a Tris-HCl/Tricine buffer system, and by gel filtration on a Sephadex G-150 column.

Enzyme Assays

Amidolytic activity of the mesquite enzyme was measured with Bz-DL-Arg-pNA (1.0 mM), Suc-Ala-Ala-Pro-Phe-pNA, Suc-Ala-Ala-Ala-pNA, MeO-Suc-Arg-Pro-Tyr-pNA, S2266, S2302, S2251, S2222, S2238, and S2484 (each at 0.2 mM) in 1.0 ml of 0.1 M Tris-HCl, pH 8.0, 0.15 M NaCl, 5 mM CaCl₂. For routine assays, pH optimal determination, temperature effects, and the effects of inhibitors on enzyme activity, Bz-DL-Arg-pNA was used as substrate.

Enzyme Specificity

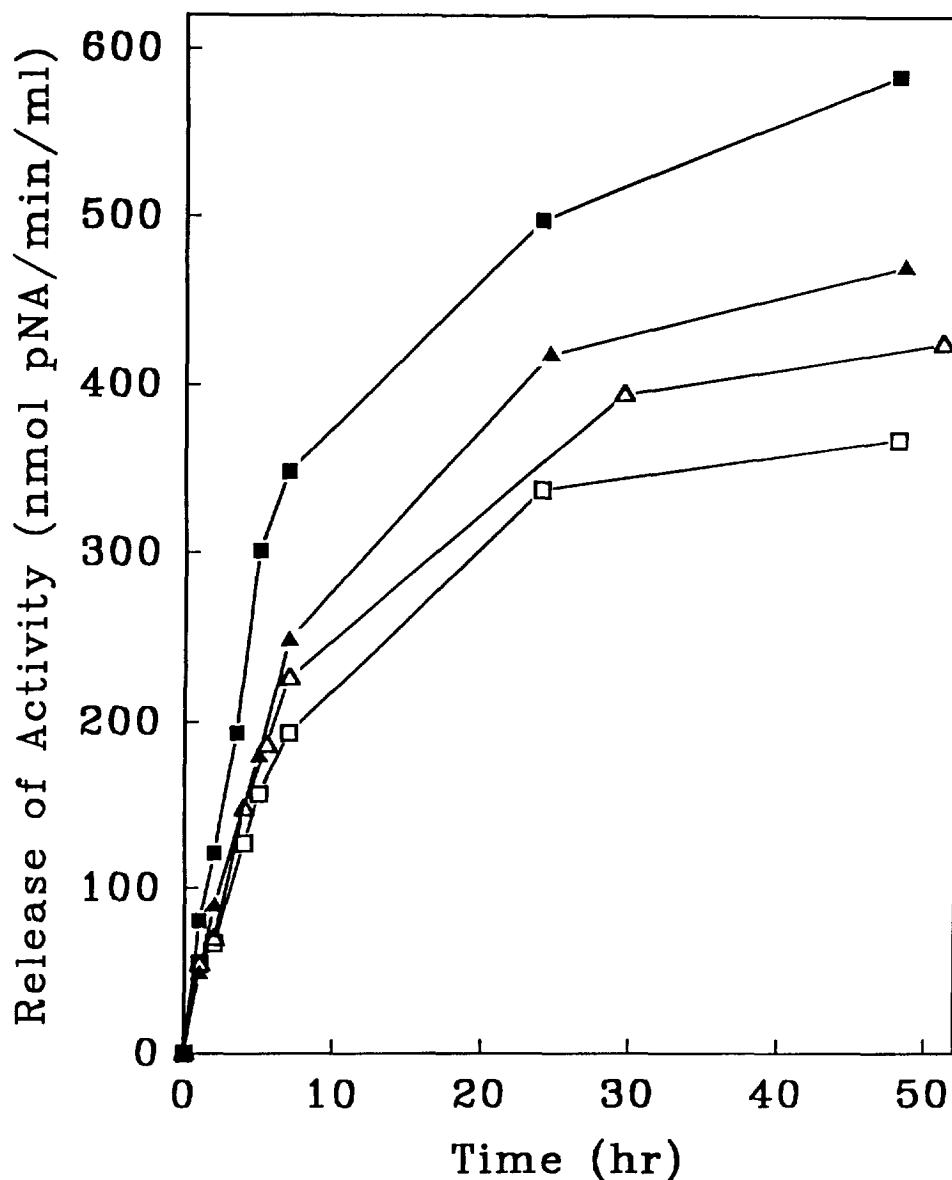
The purified enzyme (2.5 μ g, 27.8 nM) was incubated with a variety of peptides in 0.1 M Tris-HCl, pH 8.0, 0.15 M NaCl, 5 mM CaCl₂, 0.125% sodium azide at 37°. Aliquots were removed at various time intervals, and digestion was stopped by quick-freezing in dry ice. Each reaction mixture was subjected to high performance liquid chromatography, using an Ultrasphere ODS reverse-phase column (4.6 mm $\times$ 25 cm, 5 μ m) (Beckman Instruments) and a linear gradient from 0.1% trifluoroacetic acid to 0.08% trifluoroacetic acid containing 80% acetonitrile over a 60-min period (1.0 ml/min). Peptides were detected at 220 nm, collected, and analyzed for amino acid content. The enzyme:substrate molar ratios used were as follows: VIP (1:250), neurotensin (1:500), substance P (1:600), bradykinin or Met-Lys-bradykinin (1:700), dynorphin fragment 1-13 (1:1400), ANP (1:3000), and angiotensin II (1:7000).

Results

Enzyme Purification

When mesquite pollen was stirred in the cold with buffer, Bz-Arg-pNA activity was readily released (Figure 1). Most enzyme (60%) was solubilized within the first 7 h; however, appreciable additional activity had accumulated by 24 h. Ex-

Figure 1. Release of proteolytic activity from mesquite pollen with time. Mesquite pollen, 1.0 g in 10 ml (1:10, wt/vol) or 2.5 ml in 10 ml (1:4, wt/vol) of Bis-Tris, pH 6.5–5 mM CaCl_2 (Buffer A) or 0.1 M Tris-HCl, pH 8.0–0.15 M, 15 M NaCl–5 mM CaCl_2 (Buffer B) was stirred in the cold. At the times indicated, 1.0 ml was removed and centrifuged for 5 min in a microcentrifuge and the supernatant assayed with Bz-DL-Arg-pNA. ■, Buffer A, 1:4; ▲, Buffer B, 1:4; △, Buffer B, 1:10; □, Buffer A, 1:10.



traction with Bis-Tris buffer for 48 h gave the highest amount of soluble activity, and this was utilized routinely to obtain a crude extract.

As shown in Table 1, the purification required several steps with a final yield of 12%, although yields of up to 20% were occasionally obtained. Significantly, affinity chromatography using either benzamidine-Sepharose, lysine-Sepharose, or arginine-Sepharose was unsuccessful, all Bz-Arg-pNA activity passing unretarded through columns containing these materials. Although the endopeptidase also was not bound to CBS, this was an important step because much contaminating protein was adsorbed by this dye-conjugate together with virtually all of the yellow pigment present in the initial extract. The final enzyme preparation was quite stable, retaining activity for at least 3 mo at -20° and even after frequent freezing and thawing. Ca^{2+} (1.0 mM) was necessary for the retention of activity although it had no effect on enzyme activity.

Physical Properties

Treatment of the purified enzyme with NaDodSO₄, followed by gel electrophoresis, revealed a single band with a molecular mass of 84 kD (Figure 2). This band occurred under both reducing and nonreducing conditions, suggesting that it was composed of a single polypeptide chain. This was confirmed by Sephadex G-150 gel filtration although the molecular mass calculated was slightly higher (94 kDa). Amino terminal sequence analysis gave a negative result, indicating that the purified enzyme had a blocked amino-terminus. The amidase activity of the enzyme showed a broad pH optimum from pH 7.5 to 9.0 but rapidly lost activity at either higher or lower pH values. Although an acid pH precipitation step was utilized during its purification, the length of time at which the partially purified enzyme was kept at this pH was short enough to minimize any activity loss. It was also stable at 37° for at least 48 h although it lost some activity (50%) after 6 h at 50° and all activity within 15 min at 60° .

TABLE 1
Purification of mesquite pollen trypsin-like proteinase*

Fractionation Step	Total Activity	Total Protein	Specific Activity	Purification	Yield
	U†	mg	U/mg	Fold	Percent
Crude extract	103,457	11,942	8.66	1	100
(NH ₄) ₂ SO ₄ , 30 to 60%	49,956	3,817	13.09	1.5	49
pH 4.5 supernatant	41,548	2,334	17.80	2	41
Cibacron blue-sepharose	34,890	584	59.74	7	34
DEAE-Sephacel	33,120	214	154.77	18	32
Phenyl sepharose	31,785	65	489.00	531	31
Sephacryl S-100	14,060	2	6,390.91	738	14
Mono Q FPLC	12,866	0.56	22,975.00	2,653	12

* Based on 100 g of pollen.

† Based on enzymatic activity using Bz-L-Arg-pNA where 1 U = nmol pNA released/min.

Amidase and Peptidase Specificity

The purified enzyme was tested for its amidolytic activity on a number of synthetic peptide nitroanilides. As shown in Table 2, only those substrates containing either a P₁-arginine or lysine were cleaved, with the highest rate being for Bz-Arg-pNA, followed by S-2266. All others were hydrolyzed significantly more slowly.

With peptide substrates, there was rapid hydrolysis after arginine residues, particularly with angiotensin II and ANP (Figure 3), which were both completely converted to smaller fragments after 1-h incubation at low E:S ratios (1:7000 for

angiotensin II and 1:3000 for ANP). Other peptides tested were also fragmented, albeit more slowly and with higher E:S ratios. For example, 50% of bradykinin (or Met-Lys-bradykinin) and neurotensin were hydrolyzed after 2 h, using 1:700 and 1:500 E:S ratios, respectively. However both VIP and substance P were poor substrates under the conditions used. Angiotensin II (11), ANP (12), neurotensin (13), bradykinin (14), and VIP (15) were all inactivated by cleavage at the sites shown in Table 3. The activity of substance P would not have been affected since cleavage occurring up to residue four from the amino terminus does not inactivate this peptide (16). Cleavage after lysine residues in peptides was

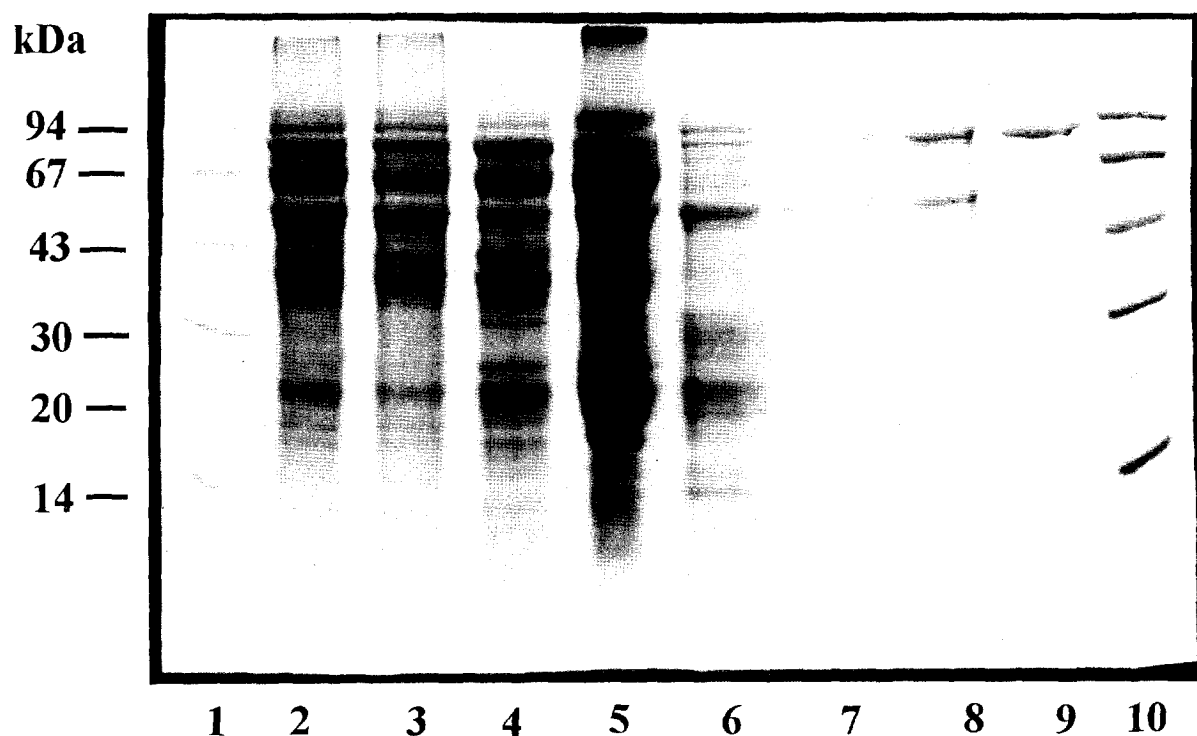


Figure 2. SDS-PAGE of mesquite pollen arginyl endopeptidase at various purification. Lanes 1 and 10: molecular weight markers (phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 23 kDa; α -lactalbumin, 14 kDa). Lane 2: pollen extract. Lane 3: ammonium sulfate precipitate. Lane 4: acid supernatant. Lane 5: CBS wash. Lane 6: DEAE Sephacel eluate. Lane 7: phenyl sepharose eluate. Lane 8: Sephacryl S-100 wash. Lane 9: FPLC Mono Q eluate. The gel was stained with Coomassie Blue R-250.

TABLE 2
Amidolytic activity of mesquite pollen proteinase

Substrate	Activity nmol/ μ g/min
Bz-Arg-pNA-(BAPNA)	1,388.7
D-Val-Leu-Arg-pNA (S2266)	882.6
D-Pro-Phe-Arg-pNA (S2302)	337.2
D-Val-Leu-Lys-pNA (S2251)	337.2
Bz-Ile-Glu (γ -OR)-Gly-Arg-pNA (S2222)	281.2
D-Phe-Pip-Arg-pNA (S2238)	70.3
Glu-Pro-Val-pNA (S2484)	0
Suc-Ala-Ala-Ala-pNA	0
Suc-Ala-Ala-Pro-Phe-pNA	0
MetO-Suc-Arg-Pro-Tyr-pNA	0

much slower, even at very high E:S ratios (e.g., 1:300). Thus, hydrolysis of peptide bonds after this residue may not be of any physiological significance.

Protein Digestion

While it was initially assumed that the purified arginyl endopeptidase was a proteinase, it was found to degrade large proteins such as casein rather poorly. Activation of some proenzymes such as prekallikrein and prothrombin did occur, but the rate was slow and the molar ratios of endopeptidase to zymogen that were required were rather high (1:30 for prothrombin and 1:1 for prekallikrein in both *in vitro* and *in vivo* assays). The *in vivo* studies involved the use of a vascular permeability enhancement assay in guinea pigs (17),

which indicated the release of bradykinin through activation of the kallikrein system. However, significantly higher concentrations of enzyme were required to generate activity equal to that found with either the dust mite serine proteinase (5 $\times$) (7) or the cysteine proteinase arg-gingipain (100 $\times$) (18) (data not shown), suggesting that this enzyme was unlikely to be involved in activation of the kallikrein/kinin pathway. Taken together, these data suggest that the enzyme should probably be considered an arginyl endopeptidase rather than a proteinase.

Inhibition Spectrum

Using Bz-Arg-pNA as substrate, it was found that the mesquite pollen endopeptidase was inhibited strongly by both DFP and AEBSF and weakly by PMSF and 3,4-dichlorisocoumarin (Table 4). It was also inhibited by benzamidine, TLCK, antipain, leupeptin, and D-Phe-Pro-Arg-CH₂Cl. However, activity was not lost by treatment with iodoacetamide, EDTA, or TPCK. Clearly, these data indicate that the endopeptidase is a member of the serine proteinase class of enzymes, with a specificity similar to trypsin.

Significantly, none of the available plant trypsin inhibitors, including soybean trypsin inhibitor, lima bean trypsin inhibitor, tomato inhibitors I and II, potato inhibitors PC 1 and 3, and tobacco leaf inhibitor I were effective in abolishing enzyme activity. A similar negative result was also found with other natural trypsin inhibitors, including aprotinin, ovomucoid, and any of the inhibitors in human plasma. Enzyme activity was also not affected by the presence of H₂O₂ (100 mM), indicating an insensitivity to oxidizing agents.

Figure 3. Decrease in percentage of peak area of various intact bioactive peptides from HPLC with time after incubation with the pollen arginyl endopeptidase. Purified mesquite pollen arginyl endopeptidase was incubated with VIP, neurotensin, substance P, bradykinin, ANP, and angiotensin II in 0.1 M Tris-HCl, pH 8.0–0.15 M NaCl–5 mM CaCl₂–0.125% sodium azide at 37° C, as described in MATERIALS AND METHODS. At various times, aliquots were removed and frozen immediately in dry ice and later, peaks were separated on HPLC. ●, VIP, 1:300 enzyme to peptide molar ratio; △, neurotensin, 1:500; □, substance P, 1:600; ○, bradykinin, 1:700; ▲, ANP, 1:3000; ■, angiotensin II, 1:7000.

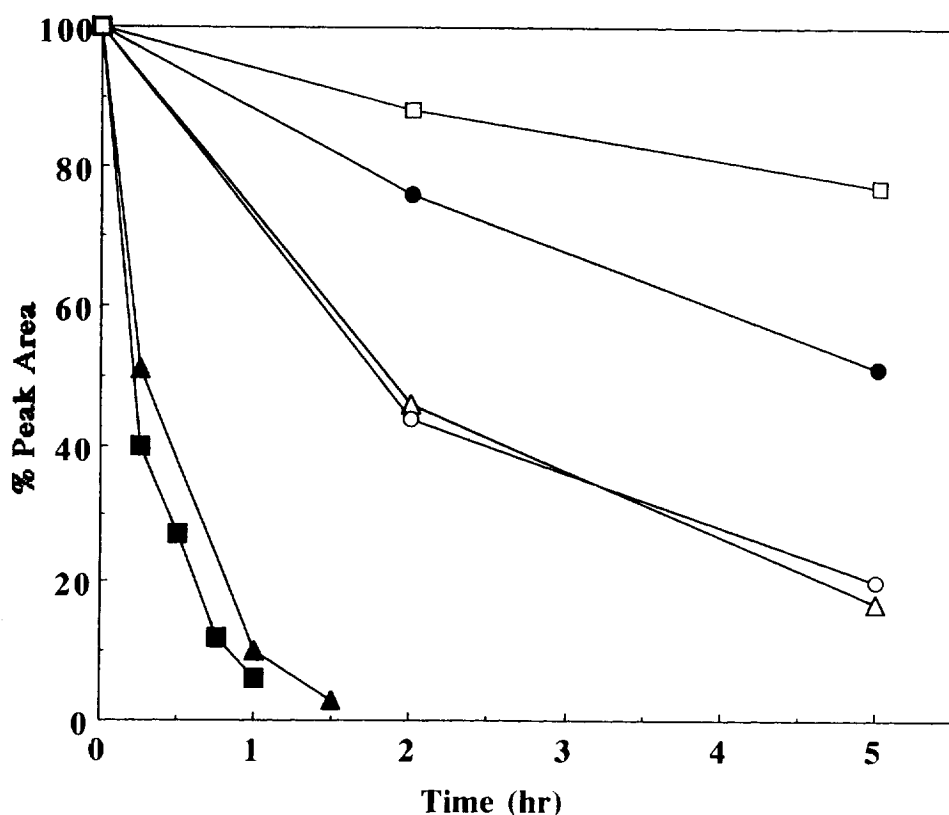


TABLE 3
Specificity of cleavage of peptides of known sequence by
mesquite pollen arginyl peptidase

Peptide	Sequence
Angiotensin II	D-R-V-Y-I-H-P-F
Atrial natriuretic peptide	S-L-R-R-S-S-C-F-G-G-R-M-D-R-I-G-A-Q-S-G-L-G-C-N-S-F-R-Y
Bradykinin	R-P-P-G-F-S-P-F-R
Neurotensin	E-L-Y-E-N-K-P-R-P-Y-I-L
Vasoactive intestinal peptide	H-S-D-A-V-F-T-D-N-Y-T-R-L-R-K-Q-M-A-V-K-K-Y-L-N-S-I-L-N
Substance P	R-P-K-P-Q-Q-F-F-G-L-M
Dynorphin fragment 1-13	Y-G-G-F-L-R-R-I-R-P-L-K

TABLE 4
Effect of inhibitors on the amidolytic activity of mesquite pollen proteinase

Pre-Incubation Time	Inhibitor	Concentration	Residual Activity
10 min	DFP	0.01 mM	55.2
		0.1 mM	0.0
24 h	PMSF	2.0 mM	71.3
10 min	AEBSF	1.0 mM	14.7
24 h	3,4 Dichloroisocoumarin	0.1 mM	51.9
		0.4 mM	21.8
10 min	Iodoacetamide	10 mM	103.0
10 min	EDTA	4 mM	115.0
10 min	Benzamidine	10 mM	41.0
10 min	TLCK	2.8 μ M	27.5
		28.0 μ M	0.0
10 min	TPCK	1 mM	102.9
5 min	Antipain	8.3 nM	32.7
		82.7 nM	0.0
5 min	Leupeptin	0.4 μ M	13.8
		4.2 μ M	0.9
10 min	SBTI	0.5 mg/ml	118.5
10 min	LBTI	0.5 mg/ml	113.3
10 min	Aprotinin	0.5 mg/ml	104.3
10 min	Ovomucoid	0.5 mg/ml	114.5
10 min	D-Phe-Pro-Arg-CH ₃ Cl	0.2 μ M	65.6
		2.0 μ M	23.2
		20 μ M	0.0

Discussion

Pollen is known to be a significant triggering factor for the development of both allergic responses and asthma. However, until now most studies focused on the isolation of specific allergens and a determination of their immunologic properties. Indeed, little attention has been paid to the fact that many of the pollen allergens are enzymes which have, for example, lyase (19), esterase (20), and polygalacturonase (2) activities. Even the grass allergen, Lol PI, has a resemblance to members of the cysteine proteinase family although no enzymatic activity for this major allergen has yet been found (R. Pike and J. Travis, unpublished observations). More recently (21), it has been demonstrated that the major mite allergen, Der PI, is a serine proteinase that is also active in the generation of bradykinin through activation of the kal-

likrein/kinin system (22-24). The presence of kinins has been previously demonstrated in nasal washes after intranasal challenge with allergens (25), and proteolytic activity has been previously detected in pollen. It was, therefore, our premise that proteinases present in and released from pollen within the airways might be involved in the generation of kinins that would then cause local inflammatory reactions. After examining over twenty pollen sources, we chose to examine both mesquite and ragweed pollens as sources of proteinase since they had considerably more Bz-Arg-pNA activity than any of the other pollens and, in addition, also contained chymotrypsin-like activity. A report on the properties of the enzymes in ragweed pollen, together with the properties of the chymotrypsin-like enzyme from mesquite will be described separately.

The results we obtained indicated that we had a serine en-

dopeptidase with properties that would make it a member of the trypsin family. However, the enzyme was not inhibited by typical protein proteinase inhibitors of trypsin and, more significantly, also had a blocked amino-terminus, suggesting that its mechanism of action was not totally consistent with other proteinases in this class. Indeed, sequencing of the gene coding for this protein has been obtained using DNA obtained from mesquite anthers, with the results suggesting that the arginyl endopeptidase was more closely related to *Escherichia coli* protease II, a member of the prolyl endopeptidase family (H. Gibson and co-workers, unpublished data).

Perhaps the most important property of the purified mesquite proteinase, however, was its inability to degrade protein substrates while being very efficient in fragmenting small peptides, particularly angiotensin II and ANP. Both of the latter are vasoactive peptides that are antagonists of each other. Angiotensin II is a strong vasoconstricting agent (26, 27), whereas ANP causes vasodilation (28–30). However, ANP modulates rather than increases vascular permeability, so that the degradation of both peptides would ameliorate the consequences of asthma by increasing the kinin/angiotensin II ratio at localized sites. This result is similar to what is believed to occur with the mite proteinase system. However, in that case the ratio is altered by increasing kinin production at localized sites rather than decreasing angiotensin II levels, as we suggest could occur here.

During an acute allergic asthmatic attack or in chronic asthma due to the presence of allergens, airway epithelial cells, together with cilia, are stripped away (probably by proteinases as well) from the basement membrane, exposing nerve endings (31). Plasma is exuded from the pulmonary vasculature into the asthmatic airway lumen (32) and mucus is hypersecreted (33, 34). Due to the loss of cilia, there is mucociliary dysfunction (35, 36) so that the inhaled pollen grains would not be efficiently cleared and, therefore, able to release significant amounts of endopeptidase together with other proteins. In this manner, the peptidase would be in contact with neuropeptides, peptide hormones, and other bioactive peptides released from pulmonary neurons, neuroendocrine cells, inflammatory cells, or vascular endothelium (37–40) where it would be able to act to initiate and/or augment the proinflammatory reaction. Indeed, the presence of any external source of peptidase could have a dramatic effect on upsetting the balance between proinflammatory and anti-inflammatory components within the airways. Most of the pollens we have tested contained both Bz-Arg-pNA and chymotrypsin-like activity. Therefore, we are continuing our studies to determine whether either activity, individually or together, plays any role in the development of allergies and/or asthma.

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