

Purification and Characterization of a Novel Peptidase (II_{mes}) from Mesquite (*Prosopis velutina*) Pollen*

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Although the mesquite plant (*Prosopis velutina*) is not as widely distributed as some other allergenic species, its pollen can induce serious pollinosis in areas where it is localized. We previously isolated and characterized a peptidase from mesquite pollen with trypsin-like specificity (peptidase I_{mes}) (Matheson, N., Schmidt, J., and Travis, J. (1995) *Am. J. Respir. Cell Mol. Biol.* 12, 441–448). Now we have characterized a second enzyme with specificity for hydrophobic residues (mesquite pollen peptidase II_{mes}). This enzyme has a molecular mass near 92 kDa and activity that was not affected by reducing or chelating agents but was inhibited by specific synthetic serine proteinase inhibitors and the aminopeptidase inhibitor bestatin. However, it was not inhibited by human plasma proteinase inhibitors, nor did it inactivate any of those tested. The enzyme possessed amidolytic activity against *p*-nitroanilide substrates most effectively after alanine residues and also displayed aminopeptidase activity against non-*p*-nitroanilide peptides with a preference for phenylalanine. This specificity for hydrophobic amino acid residues was corroborated by inhibition studies with chloromethyl ketone and organophosphonate inhibitors. More interesting from a physiological point of view is that the bioactive peptides, angiotensins I and II and vasoactive intestinal peptide, were also hydrolyzed rapidly, indicating an ability of peptidase II_{mes} to act also as an oligopeptidase. Because these bioactive peptides play a role in the inflammatory responses in allergic asthma, our data suggest that the purified mesquite pollen peptidase II_{mes} may be involved in the degradation of neuro- and vasoactive peptides during pollen-initiated allergic reactions.

Asthma is an allergic inflammation of the lungs which can occur after allergen sensitization. Such inflammatory responses are normally meant to defend against invading organisms or particulates or to effect tissue repair and are thus beneficial; however, in asthma, the response becomes exaggerated (perhaps because of a hereditary predisposition (1)), leading to adverse effects on the airways (2). Macrophages phagocytize the allergens introduced to the lungs by exposure to various environmental irritants such as dust, pollutants, and pollen, and process them to smaller fragments. As antigen-presenting cells, they then activate T-cells (3, 4) to stimulate B-cells to produce IgE. This immunoglobulin, when bound to a specific allergen, in turn, stimulates and activates several alveolar cell types to produce the many mediators of inflammation: histamine, prostaglandins, leukotrienes, cytokines, neu-

tral proteases, active oxygen species, and chemoattractants (5). The interaction of these mediators leads to the pathology of asthma, including bronchoconstriction, hypertrophy of airway smooth muscle, vasodilation, submucosal edema, and mucus hypersecretion (6). Also, the mucociliary apparatus becomes dysfunctional, reducing the clearance of inhaled particulates. Epithelial cells lining the airways are shed during this inflammatory response, removing a protective barrier (2) and are also a source of neutral endopeptidase (which normally degrades various bronchoconstrictor peptides (7)) while exposing nerve endings (8) that secrete neuropeptides such as vasoactive intestinal peptide (VIP)¹ and substance P, and vasoactive peptides (e.g. angiotensin II). VIP, a neurotransmitter of the nonadrenergic inhibitory system (9), has an anti-inflammatory effect inhibiting lymphocyte proliferation and interleukin-2 release and is also a potent bronchodilator (10). Substance P, a neurotransmitter of the nonadrenergic excitatory system (11), in contrast, has a proinflammatory effect, increasing vascular permeability and bronchoconstriction, causing macrophages to release proinflammatory substances, and enhancing phagocytosis by neutrophils and macrophages (12). Angiotensin II is a strong vasoconstricting agent (13).

Pollen is one of the major initiators of allergic asthma. This gamete contains proteins (allergens) that are solubilized in the airway mucus and proceed to induce an immunological response. However, other proteins are also released, of which several have proven to be oligopeptidases (14–16). Because these latter enzymes appear to be members of a family of pollen oligopeptidases with varying specificities for peptide hydrolysis, we propose to name them, at least temporarily, as: peptidases I_{mes} and I_{rag} (trypsin-like specificity from both mesquite and ragweed pollens), peptidase II_{rag} (chymotrypsin-like specificity from ragweed pollen), and, as described in this report, peptidase II_{mes} (hydrophobic amino acid specificity from mesquite pollen), an enzyme that rapidly degrades VIP, angiotensin II, and its precursor, angiotensin I. We suggest that through exo- and oligopeptidase activity, pollen may have the capability for participation in the inflammatory processes in allergic asthma by mechanisms other than those involving its immunological component.

EXPERIMENTAL PROCEDURES

Materials

H-Val-*p*NA, H-Leu-*p*NA, *N*-Suc-Ala-Ala-Pro-Phe-*p*NA, *N*-Suc-Ala-Ala-Pro-Leu-*p*NA, *N*-Suc-Ala-Ala-Val-Ala-*p*NA, *N*-Suc-Ala-Ala-Ala-*p*NA, *N*-Suc-Phe-*p*NA, benzoyl-DL-Arg-*p*NA, TPCK, TLCK, iodoacet-

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¹ The abbreviations used are: VIP, vasoactive intestinal peptide; *p*NA, *p*-nitroanilide; Suc, succinyl; TPCK, tosyl-L-phenylalanine chloromethyl ketone; TLCK, *N*^α-*p*-tosyl-L-lysine chloromethyl ketone; AEBF, 4-(2-aminoethyl)benzenesulfonyl fluoride; HIV, human immunodeficiency virus; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; FPLC, fast protein liquid chromatography; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; HPLC, high performance liquid chromatography.

amide, bestatin ([[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine), angiotensins I and II, VIP, atrial natriuretic peptide, bradykinin, substance P, neurotensin, Phe-Gly-Leu-Met (substance P fragment) (peptide 1), Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg (active fragment of myelin basic protein) (peptide 2), Ala-Ser-Thr-Thr-Asn-Tyr-Thr (peptide T = HIV inhibitor) (peptide 3), and Leu-Pro-Ser-Arg (lymphocyte-activating pentapeptide from the F_c region of human IgG1) (peptide 4) were obtained from Sigma. H-Ala-pNA, H-Ala-Ala-pNA, H-Ala-Ala-Ala-pNA, Ac-Ala-pNA, Ac-Ala-Ala-pNA, H-Phe-pNA, H-Ile-pNA, H-Ala-Phe-pNA, H-Glu-Ala-pNA, N-Suc-Ala-Phe-Pro-Phe-pNA, Suc-Ala-Ala-Pro-Ala-pNA, benzyloxycarbonyl-Ala-Ala-Leu-pNA, and benzoyl-Tyr-pNA were from Bachem. Diisopropyl fluorophosphate and 3,4-dichloroisocoumarin were obtained from Calbiochem, and AEBSE and EDTA were from Boehringer Mannheim. The mesquite pollen was a kind gift from Dr. Justin O. Schmidt (Carl Hayden Bee Research Center, Tuscon, AZ). All chloromethyl ketone (except TPCK and TLCK) and organophosphonate inhibitors were kindly provided by Dr. James Powers (Georgia Institute of Technology, Atlanta).

Methods

Enzyme Extraction and Purification—Mesquite pollen (100 g) was extracted by stirring in 400 ml of 0.02 M Bis-Tris, pH 6.5, 5 mM CaCl₂ (buffer A) overnight at 4 °C. Purification of the enzyme was performed using exactly the procedures described previously (14) with ammonium sulfate fractionation, acid precipitation of contaminants, and Cibacron blue-Sepharose, DEAE-Sepharose, and phenyl-Sepharose chromatography. The active eluate from the phenyl-Sepharose column was dialyzed overnight at 4 °C against buffer A with two changes and concentrated to 20 ml using an Amicon P-30 membrane. The final step of purification involved the application of the dialyzed and concentrated enzyme solution to a Mono Q FPLC column (Amersham Pharmacia Biotech) equilibrated with buffer A. The column was washed with buffer A for 5 min, followed by a 0–0.05 M NaCl gradient for 5 min, then a 0.05–0.15 M NaCl gradient for 50 min during which the enzyme activity was eluted. The native conformation of the enzyme was obtained by polyacrylamide gel electrophoresis using a Tris-HCl/Tricine buffer system (17) omitting SDS.

Molecular Weight Determination—The molecular weight of the purified enzyme (peptidase II_{mes}) was determined by both SDS-polyacrylamide gel electrophoresis using a Tris-HCl/Tricine buffer system (17) with or without reducing conditions and by gel filtration on a Sephadex G-150 column (2.2 × 90 cm).

Enzyme Assays—For routine assays during purification, pH optimum determination, temperature effects, and the effects of inhibitors, the activity of peptidase II_{mes} was only measured spectrophotometrically at 405 nm with H-Ala-pNA (1 mM, final concentration) in either 0.2 or 1.0 ml of 0.1 M Tris-HCl, pH 8.0, 0.15% dimethyl sulfoxide at 25 °C. In inhibitor studies, the enzyme was incubated with inhibitors for 15 min at 25 °C before the substrate (H-Ala-pNA) was added. Amidolytic activity of several substrates (1 mM, final concentration) was determined in 0.2 ml of the same buffer and temperature as above. Protein concentration was determined by the bicinchoninic acid-Cu(II) sulfate procedure with bovine serum albumin as the standard (18).

Sequence Analysis—Peptidase II_{mes} (1.06 nmol) was denatured by boiling in 1% SDS followed by incubation with 0.017 nmol of high molecular weight Arg-gingipain from *Porphyromonas gingivalis* (19) in 0.2 ml of 0.02 M Tris-HCl, pH 7.6, and 1 mM fresh cysteine overnight at 37 °C. After SDS-polyacrylamide gel electrophoresis of the digest and electroelution to a polyvinylidene difluoride membrane, sequence analysis was performed with an Applied Biosystems Procise Protein sequencer using the program designed by the manufacturer.

Enzyme Specificity and Kinetics—For specificity studies, the purified enzyme (35.3–106.0 nM) was incubated with several bioactive peptides (20.0–64.0 μM) at enzyme:substrate molar ratios of 1:400–1:600 in 0.1 M Tris-HCl, pH 8.0, at 37 °C. For studies with peptides with NH₂-terminal residues of phenylalanine, alanine, and leucine, the purified enzyme (58.7–78.0 nM) was incubated with each of the substrates (64.3–143.2 μM) at enzyme:substrate molar ratios of 1:1,000–1:8,000 in the same buffer and temperature as above. Aliquots of 35 μl were removed at various times and added to 2 μl of 20% trifluoroacetic acid to stop the reaction. Each reaction mixture was subjected to high performance liquid chromatography (HPLC) using an Ultrasphere ODS reverse phase column (4.6 × 25.0 cm, 5 μm) (Beckman Instruments) and a linear gradient from 0.1% trifluoroacetic acid to 0.08% trifluoroacetic acid containing 80% acetonitrile over a 30-min period (1 ml/min). Peptides were detected at 220 nm. The same reaction mixtures were analyzed for amino acid composition by mass spectrometry. Some of the

TABLE I
Purification of mesquite pollen peptidase II_{mes}
Results are based on 100 g of pollen.

Fractionation step	Total activity ^a	Total protein	Specific activity	Purification	Yield
	units	mg	units/mg	-fold	%
Crude extract	81,700	10,200	8.0	1	100
(NH ₄) ₂ SO ₄ , 30–60%	75,800	5,520	13.7	2	93
pH 4.5 supernatant	67,800	1,450	46.7	6	83
Cibacron blue-Sepharose	36,400	374	97.3	12	45
DEAE-Sepharose	34,600	122	284.0	35	42
Phenyl-Sepharose	24,300	7.4	3280.0	410	30
Mono Q FPLC	19,700	0.72	27,400.0	3,420	24

^a Based on enzymatic activity using L-Ala-pNA where 1 unit = nmol of pNA released/min.

samples were examined by matrix-assisted laser desorption ionization. The matrix (2 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in a 50:50 mixture of water:acetonitrile with 0.1% trifluoroacetic acid) was placed on the target with approximately 0.5 μl of sample. The samples were then analyzed with a Bruker Reflex time-of-flight mass spectrometer (Billerica, MA) using matrix-assisted laser desorption ionization in linear mode, 100 shots averaged with mass range scanned from 0 to 1,500 m/z for bradykinin to 0–3,600 m/z for VIP. Some samples were analyzed by liquid chromatography-mass spectrometry using a PE-Sciex API I (atmospheric pressure ionization) plus mass spectrometer coupled with an Applied Biosystems 140 B solvent delivery system and an ABI 759A absorbance detector. The sample (20 μl) was injected onto an Asahipak ODP C18 column (1 × 250 mm, 5 μm, 200 Å) (Keystone Scientific Inc., Bellefonte, PA). The gradient used was from 0 to 100% B over 60 min at a flow rate of 40 μl/min. Solvent A was 0.1% trifluoroacetic acid in water, and solvent B was 90% acetonitrile and 10% water with 0.1% trifluoroacetic acid. The mass spectrometer was scanned from 50 to 500 U using a 0.2-U step and a 1.5-ms dwell time. The UV was monitored at 214 nm, and the signal was amplified 50 times by an Omni Amp II A (Omega Engineering Inc., Stamford, CT). The results of the mass spectrometry were in the form of a chromatographic trace with each peak having a number representing the mass of a specific fragment. Each number was entered into the computer program, Peptidemap 2.2, together with the sequence of the peptide being studied. The output obtained indicated the sequence of the peptide fragment matching the mass of the peak and was an indication of cleavage products from peptidase II_{mes} proteolysis.

$V_{\max}$ and K_m values for amino acid p-NAs were determined using substrate concentrations ranging from 18.75 to 250 μM with the final concentrations of enzyme from 2.0 to 19.1 nM in 0.1 M Tris-HCl, pH 8.0, 0.125% dimethyl sulfoxide at 25 °C. Values for the bioactive peptides were measured with substrate concentrations ranging from 10 to 82 μM, with the final concentration of enzyme from 2.2 to 25.4 nM in 0.1 M Tris-HCl, pH 8.0, 5 mM CaCl₂ at 25 °C with peptidase I_{mes} and 37 °C with peptidase II_{mes}. Aliquots of 35 μl were removed at various times and added to 2 μl of 20% trifluoroacetic acid to stop the reaction. Each sample was subjected to HPLC as described above. The increase in the peak area of the product with time was used to determine the rate of peptide cleavage. $V_{\max}$ and K_m values were determined by using Hyperbolic Regression Analysis.²

RESULTS

Enzyme Purification—Peptidase II_{mes} was readily liberated from the pollen grains by gentle stirring with buffer at 4 °C, with 50% of the activity being released by 2.5 h, and maximum activity at 6 h (data not shown). However, because the enzyme was very stable, extraction was usually performed overnight as a matter of convenience.

As shown in Table I, several steps were required to purify peptidase II_{mes}, with the scheme utilized being essentially equivalent to that performed for the isolation of peptidase I_{mes} (14). Although a single enzyme activity directed toward hydrolysis of H-Ala-pNA was obtained during all procedures up to the Mono Q FPLC step, three activities separated during this final

² The Hyperbolic Regression Analysis program, written by J. S. East-erby (University of Liverpool, U. K.), was obtained through shareware.

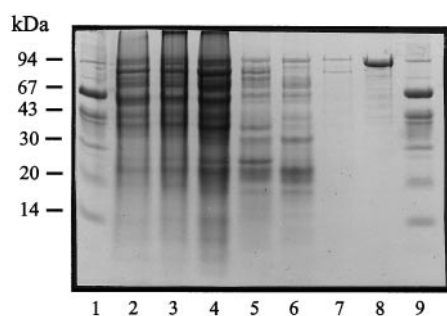


FIG. 1. SDS-polyacrylamide gel electrophoresis of mesquite pollen peptidase II_{mes} at various stages of purification. Lanes 1 and 9, molecular mass markers (rabbit muscle phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; bovine erythrocyte carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20 kDa; α -lactalbumin, 14 kDa). The following lanes contained boiled and reduced samples: lane 2, pollen extract; lane 3, ammonium sulfate precipitate; lane 4, acid supernatant; lane 5, Cibacron blue-Sepharose wash; lane 6, DEAE-Sepharose eluate; lane 7, phenyl-Sepharose eluate; lane 8, Mono Q FPLC eluate. The gel was stained with Coomassie Blue R-250.

chromatographic procedure. However, all forms exhibited identical specific activities against either H-Ala-pNA or H-Leu-pNA, all were 92 kDa, and all were inhibited by TPCK, 3,4-dichloroisocoumarin, AEBSF, or the aminopeptidase inhibitor bestatin. A native polyacrylamide gel revealed a single, diffuse, unresolvable band (data not shown). Because of these identical properties, we assumed that the various forms were isozymes of each other, pooled them together, and utilized the combined enzyme in the studies described below.

As in the case of peptidase I_{mes}, peptidase II_{mes} was also stable for at least several months at -20°C , although frequent freezing and thawing caused some loss. However, in comparison, Ca^{2+} was not required either for stability or activity.

Physical Properties—Treatment of the purified enzyme with SDS followed by gel electrophoresis revealed a major band with a molecular mass of 92 kDa and some very faint minor bands (Fig. 1). The molecular mass of the major band agreed very well with that determined by Sephadex G-150 gel filtration of active enzyme (96 kDa). Unfortunately, no amino-terminal sequence could be found, indicating that this enzyme has a blocked amino terminus. Utilizing the amidolytic activity assays with H-Ala-pNA, it was found that the enzyme had a broad pH optimum from pH 7.5 to 9.5 and was stable for at least 48 h at pH 8.0 and 25° or 37°C .

Amidase and Peptidase Specificities—Peptidase II_{mes} activity was tested with several amino acid and peptide p-NAs (Table II). H-Ala-pNA was the preferred substrate by far (and thus was used in general assays), with the next best being H-Ala-Ala-pNA. Longer peptides were even less effective as substrates. An NH_2 -terminal blocking group nearly or completely abolished activity, with Suc-Ala-Ala-Ala-pNA, Suc-Phe-pNA, Ac-Ala-pNA, and Ac-Ala-Ala-pNA not acting as substrates at all; however, there was substantial activity against the corresponding non-succinylated or non-acetylated p-NAs. Ac-Ala-pNA and Ac-Ala-Ala-pNA, in fact, acted as inhibitors at 10 and 20 times the concentration of the substrate, H-Ala-pNA. These results indicate that the amidolytic activity of peptidase II_{mes} requires a free amino group at the NH_2 terminus of a substrate, whereas a blocked NH_2 terminus can create a competitive inhibitor. It is possible that the enzyme may be sequentially removing the NH_2 -terminal amino acid or cleaving internally in the peptide pNA substrates since, as shown below utilizing non-pNA peptide substrates, both aminopeptidase and oligopeptidase activity could be detected.

The demonstration of exo- and oligopeptidase activity of peptidase II_{mes} against both bioactive and randomly selected pep-

TABLE II
Amidolytic activity of mesquite pollen peptidase II_{mes}
The assay was performed at 25°C in 0.1 M Tris-HCl, pH 8.0, 0.15% Me₂SO with 1 mM, final concentration, of the substrates above. The enzyme to substrate ratio was 1:100,000 for H-Ala-pNA and 1:25,000 for the others.

Substrate	Activity nmol pNA released / μg / min
H-Ala-pNA	26.24
H-Ala-Ala-pNA	3.51
H-Phe-pNA	1.89
H-Val-pNA	0.95
H-Ala-Ala-Ala-pNA	0.79
H-Leu-pNA	0.63
H-Ile-pNA	0.53
H-Ala-Phe-pNA	0.45
H-Glu-Ala-pNA	0.34
Suc-Met-Val-Pro-Phe-pNA	0.07
Suc-Ala-Phe-Pro-Phe-pNA	0.04
Suc-Ala-Ala-Pro-Phe-pNA	0.03
Suc-Ala-Ala-Pro-Leu-pNA	0.03
Suc-Ala-Ala-Pro-Ala-pNA	0.02
Suc-Ala-Ala-Val-Ala-pNA	0
Suc-Ala-Ala-Ala-pNA	0
Z-Ala-Ala-Leu-pNA ^a	0
Suc-Phe-pNA	0
Bz-Tyr-pNA ^b	0
Bz-Arg-pNA	0

^a Z, benzyloxycarbonyl.

^b Bz, benzoyl.

tides is given in Table III. In peptides chosen because they contained unblocked phenylalanine, leucine, or alanine residues at the NH_2 terminus, hydrolysis at the amino terminus occurred at low *E:S* molar ratios: 1:8,000 for enzyme:FGLM, 1:2,000 for LPPSR, and 1:1,000 for both FSWGAEQQR and ASTTTNYT. Internal residues of alanine and leucine were untouched at these short times of incubation and low *E:S* ratios. The enzyme was particularly effective in cleaving after NH_2 -terminal phenylalanine residues, especially in the tetrapeptide, FGLM. Hydrolysis after either the NH_2 -terminal leucine or alanine residues was much slower (Fig. 2). Thus, peptidase II_{mes} exhibited aminopeptidase activity. It is puzzling, however, why phenylalanine should be preferred rather than alanine, as was seen with the pNA substrates.

Non-pNA bioactive peptides of 8–28 amino acids were excellent substrates at *E:S* molar ratios of 1:400 to 1:600 (Table III). Angiotensins I and II were cleaved relatively rapidly (Fig. 3) with complete hydrolysis by 50 and 100 min, respectively, at these very low ratios. VIP was fragmented somewhat more slowly, 40% being cleaved by 90 min; atrial natriuretic peptide, bradykinin, substance P, and neurotensin were only slowly degraded. These results indicate that peptidase II_{mes} also has oligopeptidase activity. This is not a novel concept because multiple reports indicate that many purified enzymes have both aminopeptidase and oligopeptidase activity. These include cathepsin H (20). In addition, many peptidyl dipeptidases, including cathepsin B (21), also have oligopeptidase activity. In all cases, this has been demonstrated with peptide substrates rather than with proteins, a result that is paralleled in this study.

The hydrolysis of all bioactive peptides occurred exclusively and internally after isoleucine, leucine, phenylalanine, alanine, and methionine residues (most rapidly after isoleucine) in the substrates tested but not after every such residue in every peptide. Six of the peptides had one to three cleavages, but VIP was cleaved at seven sites. Hydrolysis after methionine residues also occurred in the dipeptides Met-Phe and Met-Tyr, which are usually used as internal standards in determining kinetic constants by HPLC. Also, a small amount of inhibition

TABLE III
Specificity of cleavage of bioactive peptides by mesquite pollen peptidase II_{mes}

Peptide	Sequence
Angiotensin I	D-R-V-Y-I-H-P-F-H-L
Angiotensin II	D-R-V-Y-I-H-P-F
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
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
Substance P	R-P-K-P-Q-Q-F-F-G-L-M
Neurotensin	E-L-Y-E-N-K-P-R-R-P-Y-I-L
Peptide 1	F-G-L-M
Peptide 2	F-S-W-G-A-E-G-Q-R
Peptide 3	A-S-T-T-T-N-Y-T
Peptide 4	L-P-P-S-R

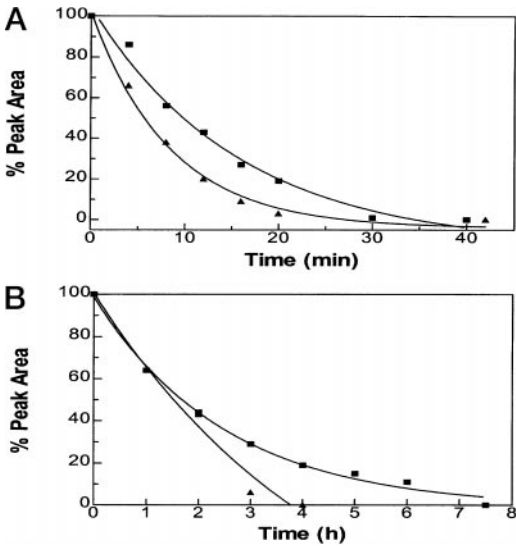


FIG. 2. Decrease in percent of peak area of various peptides with phenylalanine, leucine, or alanine in the NH₂-terminal position from HPLC after incubation with mesquite pollen peptidase II_{mes}. Purified enzyme was incubated with FGLM, FSWGAEQQR, LPPSR, or ASTTTNYT in 0.1 M Tris-HCl, pH 8.0, at 37 °C as described under "Methods." At various times, aliquots were removed and acidified to stop the reaction, and peaks were separated on HPLC. Panel A: ■, FGLM, 1:8,000; ▲, FSWGAEQQR, 1:1,000. Panel B: ■, LPPSR, 1:2,000; ▲, ASTTTNYT, 1:1,000

(20%) of the hydrolysis of the bioactive peptides occurred when Met-Phe or Met-Tyr were present. No preference for either hydrophilic or hydrophobic residues in either the P₁' or P₂ position was obvious. (The amino acid residues in substrates are numbered as P₃, P₂, P₁, etc. toward the NH₂ terminus from the cleavage site and P₁', P₂', P₃', etc. toward the COOH terminus (22).)

Although phenylalanine or alanine was the favored NH₂-terminal residue for aminopeptidase activity and isoleucine for the oligopeptidase activity, cleavage was exhibited in both cases after all three residues. Significantly, no NH₂-terminal amino acid cleavage occurred with the bioactive peptides tested because none had a suitable hydrophobic residue in that posi-

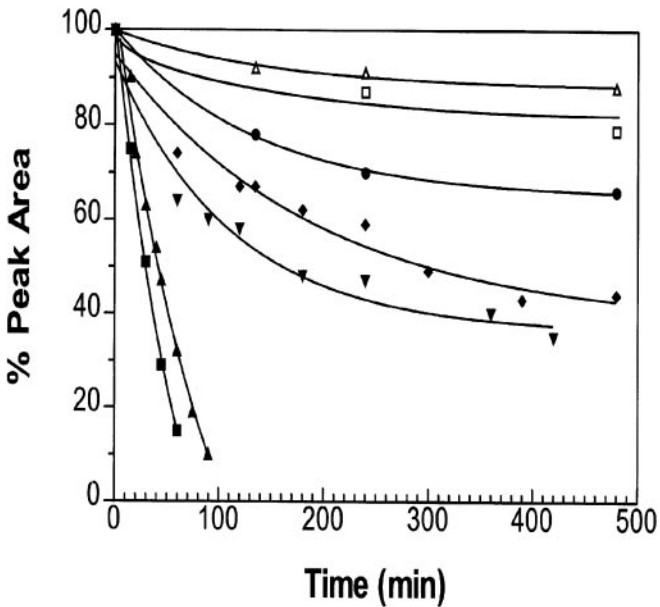


FIG. 3. Decrease in percent of peak area of various bioactive peptides from HPLC after incubation with mesquite pollen peptidase II_{mes}. Purified enzyme was incubated with angiotensins I and II, VIP, atrial natriuretic peptide, bradykinin, substance P, or neurotensin in 0.1 M Tris-HCl, pH 8.0, at 37 °C as described under "Methods." At various times aliquots were removed, acidified to stop the reaction, and peaks were separated on HPLC. ■, angiotensin I, 1:480; ▲, angiotensin II, 1:600; ▼, VIP, 1:570; ◆, atrial natriuretic peptide, 1:450; ●, bradykinin, 1:510; □, substance P, 1:400; △, neurotensin, 1:500.

tion, supporting our contention of a single enzyme with two activities of defined specificities.

As with mesquite pollen peptidase I_{mes}, peptidase II_{mes} only very slowly hydrolyzed proteins, such as azocasein and blue hide powder (a matter of days), whereas the plasma serpins, α_1 -proteinase inhibitor and α_1 -antichymotrypsin, were not hydrolyzed despite the known susceptibility to proteolytic attack within their respective reactive site loops. These results differ from data obtained recently with a chymotrypsin-like peptidase from ragweed pollen which rapidly inactivated human

TABLE IV
Effect of class-specific inhibitors on the amidolytic activity of mesquite pollen peptidase II_{mes}

Results are for a 15-min incubation at 25 °C in 0.1 M Tris-HCl, pH 8.0, with 1 mM H-Ala-pNA as substrate.

Inhibitor	Class	Concentration	Residual activity
			%
EDTA	Metallo	5 mM	100
1,10-Phenanthroline	Metallo	1 mM	44
4,7-Phenanthroline	Nonspecific	1 mM	51
Iodoacetamide	Cysteine	10 mM	100
TLCK	Cysteine/serine trypsin-like	1 mM	100
DFP ^a	Serine	0.2 mM	56
		2 mM	0
AEBSF	Cysteine/serine	1 mM	68
3,4-DCIC	Serine	0.5 mM	57
Bestatin	Aminopeptidase	15 μM	44
		120 μM	10
TPCK	Cysteine/serine chymotrypsin-like	25 μM	43

^a DFP, diisopropyl fluorophosphate.

α₁-proteinase inhibitor (15).

Inhibition Profile—Peptidase II_{mes} was not inhibited by cysteine or metalloproteinase inhibitors (Table IV) or by the specific serpins α₁-proteinase inhibitor and α₁-antichymotrypsin. It was, however, inactivated by low *M_r* serine proteinase inhibitors such as diisopropyl fluorophosphate, AEBSF, and 3,4-dichloroisocoumarin, although at rather high concentrations. As expected, TPCK was a good inhibitor, by virtue of the specificity of the enzyme toward phenylalanine residues, whereas TLCK was not inhibitory. In support of the exopeptidase activity exhibited by peptidase II_{mes}, the aminopeptidase inhibitor bestatin was very effective in reducing enzyme activity on synthetic substrates. However, both 1,10-phenanthroline (a metal chelator) and 4,7-phenanthroline (a non-chelator) inhibited as well. The non-chelating analog can bind nonspecifically to the active site of some enzymes (23), which indicates that no metal ion is involved in the enzymatic activity.

In support of the specificity of peptidase II_{mes} toward substrates with P₁ hydrophobic residues, a series of chloromethyl ketones and phosphonates was effective in blocking amidolytic activity with varying success (Table V). Significantly, the best inhibitory activity was found with an unblocked chloromethyl ketone. The concentrations of these inhibitors were much lower than the concentration of the substrate H-Ala-pNA (1:10 and lower molar ratios), indicating that this was not a competitive inhibition, in contrast to results obtained with NH₂-terminal blocked substrates, as discussed above.

Enzyme Kinetics—The kinetic activity parameters of mesquite pollen peptidases I_{mes} and II_{mes} on a variety of substrates are set forth in Table VI. H-Ala-pNA and H-Ala-Ala-pNA were again the substrates most preferred by peptidase II_{mes}. H-Leu-pNA appeared to be a better substrate than it did in Table II where activity was displayed essentially as *k*_{cat} and for H-Ala-pNA was 40 times greater than for H-Leu-pNA. However, the *K_m* of H-Leu-pNA was 20 times smaller than H-Ala-pNA and thus had a *k*_{cat}/*K_m* only slightly lower than the latter substrate. These results essentially parallel data shown earlier which were utilized in determining enzyme specificity (Table II). It should be noted that in the bioactive peptides analyzed, particularly angiotensins I and II, hydrolysis of the isoleucine-histidine peptide bond occurred five to six times faster than for H-Ile-pNA. The increased rate was apparently the result of greater affinity of the enzyme for these peptide substrates because the *k*_{cat} values were nearly the same as for H-Ile-pNA, whereas the *K_m* was six to eight times lower.

Peptidase I_{mes} also cleaved both angiotensin II and atrial

TABLE V
Effect of peptide inhibitors on the amidolytic activity of mesquite pollen peptidase II_{mes}

Results are for a 15-min incubation at 25 °C in 0.1 M Tris-HCl, pH 8.0, 0.15% Me₂SO with 1 mM H-Ala-pNA as substrate.

Inhibitor	Concentration	Residual activity
	μM	%
Ac-Ala-Ala-Pro-Ile-CH ₂ Cl	100	70
Ac-Ala-Ala-Pro-Val-CH ₂ Cl	100	69
Z-Leu-Phe ^P (OPh) ₂ ^a	100	65
Z-Ala-CH ₂ Cl	100	53
Z-Val-Val ^P (OPh) ₂	100	50
Ac-Leu-Phe-CH ₂ Cl	100	46
Z-Val ^P (OPh) ₂	100	45
Z-Ala-Val ^P (OPh) ₂	100	44
Z-Pro-Val ^P (OPh) ₂	100	40
Iso-Val-Phe-CH ₂ Cl	100	38
Z-Trp-CH ₂ Cl	100	32
Ac-Gly-Phe-CH ₂ Cl	100	31
Ac-Ala-Ala-Ala-Ala-CH ₂ Cl	100	28
Z-Phe-CH ₂ Cl	25	58
Boc-Val-Pro-Val ^P (OPh) ₂ ^b	25	43
Boc-Ala-Pro-Val ^P (OPh) ₂	25	40
Z-Gly-Leu-Phe-CH ₂ Cl	25	40
Z-Pro-Phe ^P (OPh) ₂	25	13
Ac-Ala-Leu-CH ₂ Cl	5	58
Ac-Val-Phe-CH ₂ Cl	5	52
Z-Gly-Leu-Ala-CH ₂ Cl	5	49
Ac-Ala-Ala-Phe-Ala-CH ₂ Cl	5	34
Z-Gly-Gly-Phe-CH ₂ Cl	5	30
Ac-Ala-Pro-Ala-CH ₂ Cl	5	30
Ac-Ala-Ala-Pro-Ala-CH ₂ Cl	5	17
Phe-Gly-Ala-Leu-CH ₂ Cl	5	10

^a Z, benzyloxycarbonyl.

^b Boc, butyloxycarbonyl.

TABLE VI
Kinetic parameters of mesquite pollen peptidases I_{mes} and II_{mes} with peptide substrates and comparison to other enzymes

Enzyme	Substrate	<i>k</i> _{cat}	<i>K_m</i>	<i>k</i> _{cat} / <i>K_m</i>
		s ⁻¹	μM	M ⁻¹ s ⁻¹
Peptidase II _{mes}	H-Ala-pNA	18.70	65.3	2.86 × 10 ⁵
Peptidase II _{mes}	H-Ala-Ala-pNA	8.55	37.0	2.31 × 10 ⁵
Peptidase II _{mes}	H-Leu-pNA	0.67	3.6	1.86 × 10 ⁵
Peptidase II _{mes}	H-Phe-pNA	1.21	24.8	5.22 × 10 ⁴
Peptidase II _{mes}	H-Ala-Ala-Ala-pNA	1.73	37.5	4.61 × 10 ⁴
Peptidase II _{mes}	H-Ala-Phe-pNA	0.83	23.7	3.50 × 10 ⁴
Peptidase II _{mes}	H-Ile-pNA	0.60	228.0	2.64 × 10 ³
Peptidase II _{mes}	H-Val-pNA	0.81	330.2	2.46 × 10 ³
Peptidase II _{mes}	H-Glu-Ala-pNA	0.16	175.6	9.09 × 10 ²
Peptidase II _{mes}	Angiotensin I	0.45	27.8	1.60 × 10 ⁴
Peptidase II _{mes}	Angiotensin II	0.54	38.8	1.39 × 10 ⁴
Peptidase I _{mes}	Bz-Arg-pNA ^a	28.30	135.0	2.10 × 10 ⁵
Peptidase I _{mes}	Angiotensin II	24.80	115.3	2.15 × 10 ⁵
Peptidase I _{mes}	Atrial natriuretic peptide	3.69	64.0	5.35 × 10 ⁴

^a Bz, benzoyl.

natriuretic peptide rapidly (complete hydrolysis in 60 and 90 min at 1:7,000 and 1:3,000 molar ratios, respectively (14)) but, as described previously, after an arginine residue. The *k*_{cat}/*K_m* values were similar to those found for peptidase II_{mes}, also as shown in Table VI.

Internal Sequence Comparison with That of Known Proteins—Because the NH₂ terminus of peptidase II_{mes} was blocked, making it impossible to perform a comparison with the structures of other possibly related peptidases, the enzyme was cleaved internally with *P. gingivalis* Arg-gingipain, a cysteine proteinase that hydrolyzes after arginine residues (19). Although several peptide fragments were found, one peptide specifically was obtained which had an NH₂-terminal KITFYQ-DRPDIMARYTLKIEADKYLPVELSN. Significantly, this structure had a 64% homology with the zinc-containing aminopeptidase N (membrane alanine aminopeptidase) from *Escherichia coli* and 59% homology with aminopeptidase N from

Haemophilus influenzae. The combined inhibitory activities of both 1,10- and 4,7-phenanthroline indicated the absence of a metal; however, the sequence found corresponded to residues 127–157 in aminopeptidase N from *E. coli*, whereas the zinc ion ligands in that enzyme were at residues 296, 300, and 319. Thus, this sequence was far from any of the zinc binding sites and may act like a mosaic protein as exemplified by the S8 serine peptidase from *Vibrio alginolyticus* (24), a member of the subtilisin family which acts as an endopeptidase with homologous domains similar to those found in metallopeptidases, including an aminopeptidase from *Vibrio proteolyticus* (family M28) and an endopeptidase of the thermolysin family (M4).

DISCUSSION

Pollen is one of the well known triggers of bronchial hyperresponsiveness, or exaggeration of response to inflammation, observed in allergic asthma. Although mesquite does not have a widespread distribution, it has recently been cultivated extensively in the southcentral and southwestern United States, thereby increasing its contact with people and making its pollen a serious spring aeroallergen (25). Once the pollen grains come in contact with an aqueous environment, such as the mucus layer in the lung airways, they swell and split and release many proteins (26). Pollen proteins that are allergens and elicit an immunological response have been studied abundantly (27–31). However, some of these allergens in fact appear to have, in addition, enzymatic functions displaying lyase (32), esterase (33), and polygalacturonase (34) activities. Indeed, some dust mite allergens, such as *Der p I* and *Der f I* (from *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, respectively), are cysteine proteinases, and *Der p III* and *Der f III* are serine proteinases (35).

Both mesquite and ragweed pollens have yielded peptidases with both trypsin-like and chymotrypsin-like specificities (14–16). This report concerns the results obtained in the study of a second mesquite pollen activity (peptidase II_{mes}) that was quite different from the others. The enzyme manifested both aminopeptidase and oligopeptidase activity, based on results with blocked and unblocked peptide pNAs and with unblocked polypeptides. The data obtained suggested the importance of hydrophobic residues for both activities, with phenylalanine being preferred for aminopeptidase function and multiple hydrophobic residues required in the P₁ position for internal cleavage. Such a combination of activities is not unusual and has been observed for cathepsin H (20). In addition, cathepsin B has been shown to have both peptidyl dipeptidase and oligopeptidase activities (21).

It is important to note that homology with aminopeptidases from other organisms was obtained readily in analysis of a single peptide fragment from peptidase II_{mes}. The complete amino acid sequence of peptidase I_{mes} was shown previously (14) to be homologous with protease II from *E. coli*, a member of the prolylendopeptidase family. Recent results comparing structures of a trypsin-like oligopeptidase isolated from suspension-cultured soybean cells found that homology also existed between that peptidase and prolylendopeptidases (human or porcine) including protease II (*E. coli*) (36). In fact, the soybean oligopeptidase resembled peptidase I_{mes} in other ways as well; cleavage after arginine and, to a lesser extent, lysine residues, hydrolysis of peptides only, a serine peptidase specificity, and molecular mass of 90 kDa. Whether homologies exist between peptidase II_{mes} and endopeptidases from other organisms remains to be established, but is likely.

The rapid hydrolysis of the bioactive peptides VIP and angiotensins I and II may be of potentially physiological significance. By rapidly degrading and inactivating VIP (a bronchodilator) while only slowly hydrolyzing substance P (a

bronchoconstrictor), the peptidase could be expected to exacerbate the overall bronchoconstrictive effect detected in asthmatic lungs. In addition, angiotensin II is a potent vasoconstrictor (13), and its cleavage and inactivation by the peptidase could also be expected to contribute to the overall vasodilation observed in asthmatic lungs. Kinetic rate constants indicate that the rate of cleavage of two of these peptides (angiotensins I and II) *in vitro* was relatively rapid and comparable to the rates of hydrolysis of peptide bonds by other well known proteinases, such as chymotrypsin (37).

However, the fragmenting of both angiotensins I and II into smaller peptides could have important effects of their own. Macrophages appear rapidly in the lung after local allergen challenge, suggesting a rapid migration of monocytes (38). The two tetrapeptides (DRVY and IHPF) of angiotensin II are chemotactic factors for neutrophils and, particularly, monocytes (39), which exhibit 50% of the optimal response to C5a (a potent chemotactic factor) at very low concentrations of the peptides. The cleavage of angiotensin II by peptidase II_{mes} produced a pentapeptide (DRVYI) and a tripeptide (HPF). Because the former peptide contains the chemotactic NH₂-terminal tetrapeptide it could be an effective chemotactic factor as well.

Many of the proteins extracted from pollen are enzymes that are no doubt normally involved in the germination of the plants (40). Under abnormal conditions, however, such as allergic asthma triggered by pollen, these enzymes may play a role in the pathology of the disease. Because the mesquite pollen peptidase II_{mes} with both exo- and oligopeptidase specificity described here degrades both VIP and angiotensins I and II, this enzyme may have the potential for making a significant contribution to the pathological effects observed in allergy-related asthma.

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