Purification and Characterization of a Novel Peptidase (II\textsubscript{mes}) from Mesquite (Prospis velutina) Pollen* 

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Although the mesquite plant (Prospis velutina) is not as widely distributed as some other allergenic species, its pollen can induce severe pollinosis in areas where it is localized. We previously isolated and characterized a peptidase from mesquite pollen with trypsin-like specificity (peptidase I\textsubscript{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\textsubscript{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\textsubscript{mes} to act also as an aminopeptidase.

Because these bioactive peptides play a role in the inflammatory responses in allergic asthma, our data suggest that the purified mesquite pollen peptidase II\textsubscript{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 B-cell to produce IgE. This immunoglobulin, when bound to a

EXPERIMENTAL PROCEDURES

Materials

H-Val-pNA, H-Leu-pNA, N-Suc-Ala-Ala-Pro-Phe-pNA, N-Suc-Ala-Ala-Pro-Leu-pNA, N-Suc-Ala-Ala-Pro-Val-pNA, N-Suc-Ala-Ala-Ala-pNA, N-Suc-Phe-pNA, benzoyl-\textit{t}-Arg-pNA, TPCK, TLCK, iodoacetamide, active oxygen species, and chemokina and characterizing enzyme inhibitors.

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The abbreviations used are: VIP, vasoactive intestinal peptide; pNA, p-nitroanilide; Suc, succinyl; TPCK, tosyl-L-phenylalanine chloromethyl ketone; TLCK, N\textsuperscript{\textdagger}-tosyl-L-lysine chloromethyl ketone; AEBSF, 4,4\textapos;-aminoxybenzylbenzenesulfonyl fluoride; HIV, human immunodeficiency virus; Bis-TriS, 2\textbullet\textbullet\textbullet 2-hydroxyethylamino\textbullet\textbullet\textbullet 2-hydroxyethylamino\textbullet\textbullet\textbullet 1,3-propane-1,3-diol; FPLC, fast protein liquid chromatography; Tricine, N\textbullet\textbullet\textbullet 2-hydroxy-1,1\textapos;bis(hydroxymethyl)ethyl]glycine; HPLC, high performance liquid chromatography.
amide, bestatin, (II2SR3)-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-Thr-Asn Tyr-Thr (peptide T = HIV inhibitor) (peptide 3), and Leu-Pro-Pro-Ser-Arg (lymphocyte-activating pentapeptide from the Fc region of human IgG1) (peptide 4) were obtained from Sigma. H-Ala-pNA, H-Ala-Ala-pNA, H-Ala-Ala-Ala-pNA, H-Ile-pNA, H-Glu-Ala-pNA, H-Ala-Phe-pNA, and TLCK) and organophosphonate inhibitors were kindly provided by pollen was a kind gift from Dr. Justin O. Schmidt (Carl Hayden Bee AEBSF and EDTA were from Boehringer Mannheim. The mesquite and 3,4-dichloroisocoumarin were obtained from Calbiochem, and p

**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 essentially the procedures described previously (14) with ammonium sulfate fractionation, cold precipitation of contaminants, and Cibacron blue-Sepharose, DEAE-Sephalac, and phenyl-Sepharose chromatography. The active elute 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 polycrylamide gel electrophoresis using a Tris-HCl/Tri buffer system (17) omitting SDS.

**Molecular Weight Determination**—The molecular weight of the purified enzyme (peptidase IImes) was determined by both SDS-polyacrylamide gel electrophoresis using a Tris-HCl/Tri buffer system (17) with or without reducing conditions and by gel filtration on a Sephadex G-150 column (22 × 90 cm).

**Enzyme Assays**—For routine assays during purification, pH optimum, temperature optimum, and the effects of inhibitors on the activity of peptidase IImes 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 bichromatic acid-Cu(II) sulfate procedure with bovine serum albumin as the standard (18).

**Sequence Analysis**—Peptidase IImes (1.06 nmol) was denatured by boiling in 1% SDS followed by incubation with 0.0017 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 nmol) was incubated with several bioactive peptides (20.0–64.0 μM) at enzyme-substrate molar ratios of 1:100–1:600 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 nmol) 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 ml of 20% trifluoroacetic acid to stop the reaction. The reaction mixture was subjected to high performance liquid chromatography (HPLC) using a 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 50% 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 results are based on 100 g of pollen.

| Fractionation Step | Total activitya | Total protein | Specific activity | Purification Yield |
|--------------------|----------------|--------------|------------------|--------------------|
| crude extract      | 81,700         | 10,200       | 8.0              | 1 100              |
| (NH4)2SO4, 30–60%  | 75,800         | 5,520        | 13.7             | 2 93               |
| pH 4.5 supernatant | 67,800         | 1,450        | 46.7             | 6 83               |
| DEAE-Sephacel      | 36,400         | 374          | 97.0             | 12 45              |
| Phenyl-Sepharose   | 24,300         | 7.4          | 3280.0           | 410 30             |
| Mono Q FPLC        | 19,700         | 0.72         | 27,400.0         | 3,420 24           |

* Based on enzymatic activity using H-Ala-pNA where 1 unit = nmol of pNA released/min.

**Results**

**Enzyme Purification**—Peptidase IImes 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 IImes with the scheme utilized being essentially equivalent to that performed for the isolation of peptidase Imes (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...
The demonstration of exo- and oligopeptidase activity of peptidase \( \Pi_{mes} \) against both bioactive and randomly selected peptides 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 LPSSR, and 1:100 for both FSWGAEQR and ASTTNTY. 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 \( \Pi_{mes} \) exhibited aminopeptidase activity. It is puzzling, however, why phenylalanine should be preferred rather than alanine, as was seen with the \( p/\mathrm{NA} \) substrates.

Non-\( p/\mathrm{NA} \) 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 \( \Pi_{mes} \) 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 peptidylpeptidases, 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

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**Table II**

| Substrate | Activity |
|-----------|----------|
| H-Ala-\( p/\mathrm{NA} \) | 26.24 |
| H-Ala-Ala-\( p/\mathrm{NA} \) | 3.51 |
| H-Phe-\( p/\mathrm{NA} \) | 1.89 |
| H-Val-\( p/\mathrm{NA} \) | 0.95 |
| H-Ala-Ala-\( p/\mathrm{NA} \) | 0.79 |
| H-Leu-\( p/\mathrm{NA} \) | 0.63 |
| H-Ile-\( p/\mathrm{NA} \) | 0.53 |
| H-Ala-Phe-\( p/\mathrm{NA} \) | 0.45 |
| H-Glu-Ala-\( p/\mathrm{NA} \) | 0.34 |
| Suc-Met-Val-Pro-\( p/\mathrm{NA} \) | 0.07 |
| Suc-Ala-Phe-Pro-\( p/\mathrm{NA} \) | 0.04 |
| Suc-Ala-Ala-Pro-Leu-\( p/\mathrm{NA} \) | 0.03 |
| Suc-Ala-Ala-Pro-\( p/\mathrm{NA} \) | 0.02 |
| Suc-Ala-Ala-Ala-\( p/\mathrm{NA} \) | 0 |
| Z-Ala-Ala-Leu-\( p/\mathrm{NA} \) | 0 |
| Suc-Phe-\( p/\mathrm{NA} \) | 0 |
| Bz-Tyr-\( p/\mathrm{NA} \) | 0 |
| Bz-Arg-\( p/\mathrm{NA} \) | 0 |

**Note:**
- \( Z \), benzyloxycarbonyl.
- \( Bz \), benzoyl.

**Figure 1.** SDS-polyacrylamide gel electrophoresis of mesquite pollen peptidase \( \Pi_{mes} \) 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; \( \alpha \)-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, Cibacon 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.

**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-\( p/\mathrm{NA} \), 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 \( \Pi_{mes} \) activity was tested with several amino acid and peptide \( p/\mathrm{NAs} \) (Table II). H-Ala-\( p/\mathrm{NA} \) was the preferred substrate by far (and thus was used in general assays), with the next best being H-Ala-Ala-\( p/\mathrm{NA} \). Longer peptides were even less effective as substrates. An NH\(_2\)-terminal blocking group nearly or completely abolished activity, with Suc-Ala-Ala-Ala-\( p/\mathrm{NA} \), Suc-Phe-\( p/\mathrm{NA} \), Ac-Ala-\( p/\mathrm{NA} \), and Ac-Ala-Ala-\( p/\mathrm{NA} \) not acting as substrates at all; however, there was substantial activity against the corresponding non-succinylated or non-acetylated \( p/\mathrm{NAs} \). Ac-Ala-\( p/\mathrm{NA} \) and Ac-Ala-Ala-\( p/\mathrm{NA} \), in fact, acted as inhibitors at 10 and 20 times the concentration of the substrate, H-Ala-\( p/\mathrm{NA} \). These results indicate that the amidolytic activity of peptidase \( \Pi_{mes} \) 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 \( p/\mathrm{NA} \) substrates since, as shown below utilizing non-\( p/\mathrm{NA} \) peptide substrates, both aminopeptidase and oligopeptidase activity could be detected.

As in the case of peptidase \( \Pi_{mes} \), peptidase \( \Pi_{mes} \) 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.

Utilizing the amidolytic activity assays with H-Ala-\( p/\mathrm{NA} \), Suc-Ala-Ala-\( p/\mathrm{NA} \), Suc-Phe-\( p/\mathrm{NA} \), Ac-Ala-\( p/\mathrm{NA} \), and Ac-Ala-Ala-\( p/\mathrm{NA} \) not acting as substrates at all; however, there was substantial activity against the corresponding non-succinylated or non-acetylated \( p/\mathrm{NAs} \). Ac-Ala-\( p/\mathrm{NA} \) and Ac-Ala-Ala-\( p/\mathrm{NA} \), in fact, acted as inhibitors at 10 and 20 times the concentration of the substrate, H-Ala-\( p/\mathrm{NA} \). These results indicate that the amidolytic activity of peptidase \( \Pi_{mes} \) 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 \( p/\mathrm{NA} \) substrates since, as shown below utilizing non-\( p/\mathrm{NA} \) peptide substrates, both aminopeptidase and oligopeptidase activity could be detected.

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(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 P\textsuperscript{1} or P\textsubscript{2} position was obvious. (The amino acid residues in substrates are numbered as P\textsubscript{3}, P\textsubscript{2}, P\textsubscript{1}, etc. toward the NH\textsubscript{2} terminus from the cleavage site and P\textsuperscript{1}, P\textsubscript{2}, P\textsubscript{3}, etc. toward the COOH terminus (22)).

Although phenylalanine or alanine was the favored NH\textsubscript{2}-terminal residue for aminopeptidase activity and isoleucine for the oligopeptidase activity, cleavage was exhibited in both cases after all three residues. Significantly, no NH\textsubscript{2}-terminal amino acid cleavage occurred with the bioactive peptides tested because none had a suitable hydrophobic residue in that position, supporting our contention of a single enzyme with two activities of defined specificities.

As with mesquite pollen peptidase I\textsubscript{mes}, peptidase II\textsubscript{mes} only very slowly hydrolyzed proteins, such as azocasein and blue hide powder (a matter of days), whereas the plasma serpins, a\textsubscript{1}-proteinase inhibitor and a\textsubscript{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 III**

| 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       | H-S-D-A-V-F-T-D-N-Y-T-R-L-R-K-Q-M-A-V- |
| intestinal peptide | K-K-Y-L-N-S-I-L-N               |
| Vasoactive       | S+L+R+R+S+G+G+R+M+D+R+I+G+Q+S+G-   |
| intestinal peptide | 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+F+F+P+F+G+L-M           |
| Neurotensin      | E+L+Y+E+N+K+P+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+Y+T                       |
| Peptide 4        | L+F+P+S+R                         |

**FIG. 2.** Decrease in percent of peak area of various peptides with phenylalanine, leucine, or alanine in the NH\textsubscript{2}-terminal position from HPLC after incubation with mesquite pollen peptidase I\textsubscript{mes}. Purified enzyme was incubated with FGLM, FSWGAE-GQR, LPSSR, 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; ▲, FSWGAE-GQR, 1:1,000. **Panel B:** ■, LPSSR, 1:2,000; ▲, ASTTTNYT, 1:1,000

**FIG. 3.** Decrease in percent of peak area of various bioactive peptides from HPLC after incubation with mesquite pollen peptidase I\textsubscript{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.


**Peptidase IImes from Mesquite Pollen**

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-Phenanthrolone | Metallo | 1 mM          | 44%              |
| 4,7-Phenanthrolone | Non specific | 1 mM          | 51%              |
| Iodoacetamide | Cysteine | 10 mM         | 100%             |
| TLCK      | Cysteine/serine trypsin-like | 1 mM         | 100%             |
| DFPa      | Serine  | 0.2 mM        | 56%              |
| AEBSF     | Cysteine/serine | 2 mM        | 0%               |
| 3,4-DCIC   | Serine  | 0.5 mM        | 57%              |
| Bestatin  | Aminopeptidase | 15 μM    | 44%              |
| TPCK      | Cysteine/serine | 120 μM    | 10%              |
|           |        | 25 μM         | 43%              |

a DFP, diisopropyl fluorophosphate.

**Inhibition Profile**—Peptidase IImes was not inhibited by cysteine or metalloproteinase inhibitors (Table IV) or by the specific serpins α1-proteinase inhibitor and α1-antichymotrypsin.

As expected, TPCK was a good inhibitor, by virtue of the specificity of the enzyme toward phenylalanyl residues, whereas TLCK was not inhibitory. In support of the exopeptidase activity exhibited by peptidase IImes, the aminopeptidase inhibitor bestatin was very effective in reducing enzyme activity, although at rather high concentrations.

Because the NH2 terminus of peptidase IImes was blocked, making it impossible to perform a comparison with the blocked substrate, as discussed above.

**Enzyme Kinetics**—The kinetic activity parameters of mesquite pollen peptidases Imes and IImes 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 IImes. H-Leu-pNA appeared to be a better substrate than it did in Table II where activity was displayed essentially as kcat for H-Ala-pNA was 40 times greater than for H-Leu-pNA. However, the Km of H-Leu-pNA was 20 times smaller than H-Ala-pNA and thus had a kcat/Km 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 kcat values were nearly the same as for H-Ile-pNA, whereas the Km was six to eight times lower.

Peptidase Imes also cleaved both angiotensin II and atrial 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 kcat/Km values were similar to those found for peptidase IImes, also as shown in Table VI.

**Internal Sequence Comparison with That of Known Proteins**—Because the NH2 terminus of peptidase IImes 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 protease that hydrolyzes after arginine residues (19). Although several peptide fragments were found, one peptide specifically was obtained which had an NH2-terminal KITFYQDRPDIMARYTLKIEADKYLYPVELSN. 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...
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*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 hyper-responsiveness, 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\textsubscript{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 P\textsubscript{1} position for internal cleavage. Such a combination of activities is not usual and has been observed for cathepsin H (20). In addition, cathepsin B has been shown to have both peptidylaldepeptidase 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\textsubscript{mes}. The complete amino acid sequence of peptidase II\textsubscript{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 II\textsubscript{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\textsubscript{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 vasconstrictr (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 (DRVYI and IYPY) 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\textsubscript{mes} produced a pentapeptide (DRVYI) and a tripeptide (HPF). Because the former peptide contains the chemotactic NH\textsubscript{2}-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\textsubscript{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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