Matrix Metalloproteinase Homologues from Arabidopsis thaliana

EXPRESSION AND ACTIVITY*

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Five genes potentially encoding novel matrix metalloproteinases (MMPs) have been identified on the Arabidopsis thaliana data base. The predicted proteins have a similar domain structure to mammalian MMP-7, with a propeptide and catalytic domain but no C-terminal hemopexin-like domain. Four of the A. thaliana MMPs (At-MMPs) have a predicted C-terminal transmembrane domain. The At-MMPs are differentially expressed in flower, leaf, root, and stem tissues from 14-day-old plants. The cDNA for one of the At-MMPs (At1-MMP) was cloned and expressed in Escherichia coli. Following refolding and purification, the proenzyme At1-MMP was shown to undergo autolytic activation in the presence of an organomercurial with a concomitant decrease in Mr. In contrast to this, trypsin-treatment led to the formation of an inactive product. The activated At1-MMP digested myelin basic protein, but was unable to digest gelatin or casein. Three peptide substrates for MMPs were also cleaved by At1-MMP. The enzyme activity of At1-MMP was inhibited by human tissue inhibitors of metalloproteinases 1 and 2 and the hydroxamate inhibitor BB-94.

The matrix metalloproteinases (MMPs) are a family of at least 20 zinc-dependent endoproteases in vertebrates, capable of degrading extracellular matrix substrates; they have been divided into subgroups according to their structure and function. The MMPs have a common domain structure with a signal peptide, a propeptide, a catalytic domain, a hinge region, and a C-terminal domain. The propeptide contains an invariant Cys residue that ligates the active site zinc ion to maintain latency; the catalytic domain contains a HEGHXXGXXH zinc-binding sequence characteristic of the metzincin superfamily of proteinases, followed by an invariant Met that is involved in a structural feature called the “Met-turn.” In all family members except matriplysin (MMP-7) a hinge region links to a hemopexin-like C-terminal domain that is thought to be involved in substrate specificity and binding of inhibitors. Individual MMPs contain variations on this theme: MT-MMPs (MMPs 14–17) have a transmembrane domain and cytoplasmic tail at the C terminus and, in common with MMP-11, contain a potential furin-cleavage site within the propeptide; the gelatinases (MMP-2 and -9) have an insert of three fibronectin type II repeats in the catalytic domain; and MMP-9 has a collagen-like sequence at one end of the catalytic domain (1).

Invertebrates have also been shown to possess proteinases homologous to MMPs; these include the envelysins, which are involved in the hatching process in sea urchins (2). A report has described three sequences in Caenorhabditis elegans that appear to correspond to MMPs (3). In 1991, soybean leaves were shown to contain a metalloproteinase, later shown to be homologous to MMPs. This enzyme was expressed only in adult leaves, and Southern blot analysis demonstrated a single copy gene; activity was demonstrated against a synthetic MMP substrate, and this activity was inhibited by mammalian TIMP-1 (4–6).

The plant Arabidopsis thaliana is an important model species for the study of plant biology, with many of the tools and reagents in place to manipulate genes in vivo. The relatively small size of its genome has led to a project to sequence its five chromosomes; our study began with the discovery within this project that a sequence on chromosome 4 of the A. thaliana genome codes for a protein with homology to both the soybean MMP and the vertebrate MMP family (7). During our studies, four other sequences with homology to this first one have been identified on the data base; we have named these At1-MMP to At5-MMP in order of our discovery of them on the computer (see below). Here, we examined expression of At1- to At5-MMP in tissues from A. thaliana and then cloned and expressed At1-MMP in Escherichia coli and examined its ability to cleave both protein and synthetic substrates and to be inhibited by known MMP inhibitors.

EXPERIMENTAL PROCEDURES

RT-PCR—Total RNA from 14-day-old A. thaliana plant tissues was a kind gift from Dr. M. Torres (John Innes Centre, Norwich, United Kingdom). cDNA was produced from each of flower, leaf, root, and stem RNA using Superscript II reverse transcriptase (Life Technologies) and oligo(dT) primers (Amersham Pharmacia Biotech). Because the genes for the At-MMPs have no intron sequence, the absence of genomic DNA contamination in the RNA samples was verified by treatment with RNase-free DNase (Promega) prior to the RT reaction. Primers for RT-PCR of At-MMPs were designed using the Wisconsin Package (GCC), Madison, Wisconsin, to give differing size products for each of At1- to At5-MMP and no cross-hybridization between these cDNAs (Table I). PCR was performed as follows: cycle 1, 94 °C for 2 min; cycle 2, 94 °C for 1 min, 55 °C for 90 s and 72 °C for 90 s; cycle 3, 72 °C for 10 min. Cycle 2 was repeated between 17 and 35 times, and for all primer pairs, experiments were carried out by sampling across these cycles to verify that signal from the product was within the linear range. PCR using At1-MMP primers was performed in 10 mM Tris-HCl, pH 8.8, 75 mM KCl, 1.5 mM MgCl2, whereas PCR using UBQ5 primers (a kind gift from Dr. N. Aarts, John Innes Centre) was performed in 67 mM Tris-HCl, pH 8.8, 16 mM (NH4)2SO4, 0.01% Tween 20, 1.5 mM MgCl2. Products were separated on 2% agarose gels in 1X Tris borate-EDTA.

Expression and Purification of At1-MMP—cDNA for At1-MMP was...
amplified by RT-PCR from *A. thaliana* mRNA (source as above) using the following primers: 5′-ACTCGGATCCGCTAGAGAATCACCCGGGGAAG-3′ (forward); 5′-GCGAATTCTAACATGAACTGATACGG-3′ (reverse 1); 5′-GCGAATTCTAACATGAACTGATACGG-3′ (reverse 2); and 5′-GCGAATTCTAACATGAACTGATACGG-3′ (reverse 3). Using these primers and the high fidelity *Pfu* polymerase (Stratagene), sequence was amplified to give coding regions between Ala-28 and Gly-292 (reverse 1), Leu-297 (reverse 2), or Arg-317 (reverse 3), flanked by a 5′ BamHI site and a 3′ EcoRI site. These cDNAs were then subcloned into pRSETA (Invitrogen) using standard techniques, and the reading frame was verified by sequencing. All three plasmids were transformed into *E. coli* BL21(DE3)pLysS for expression. Pilot scale expression was performed in LB containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C using 1 mM isopropyl-β-D-galactopyranoside (Melford Laboratories, Ipswich, United Kingdom) to induce expression for 1–24 h. These experiments demonstrated that the Ala-28 to Gly-292 construct gave the highest level of expression, expressing the recombinant At1-MMP as an insoluble protein within inclusion bodies. Hence, this construct was chosen for expression in a 3-litre volume using a 1-h induction period; bacteria were harvested by centrifugation, and the pellets were stored at −20 °C until extraction and purification. A pellet from 1.5 liters of culture was resuspended in 50 ml of ice-cold 10 mM Tris-HCl, pH 8.5, containing 1 mM EDTA and Complete (Roche Molecular Biochemicals) EDTA-free trypsin inhibitor. followed by inhibition of trypsin with at least a 5-fold excess of soybean trypsin inhibitor.

**Activity Assays**—The activity of At1-MMP against synthetic quenched fluorescent peptide substrates was determined by incubation either alone or with 1 mM APMA at 37 °C for up to 4 h. Samples were also treated with trypsin at a final concentration of 1 μg/ml for up to 1 h, followed by inhibition of trypsin with at least a 5-fold excess of soybean trypsin inhibitor.

**Results and Discussion**

**Matrix Metalloproteinase Homologues from *A. thaliana*—**A search of the *A. thaliana* data base reveals five DNA sequences (four genomic sequence only, one genomic sequence and cDNA) within which an open reading frame predicts a protein with homology to the vertebrate matrix metalloproteinase family. Because function is currently unknown for these enzymes, we have named them At-MMPs (for *A. thaliana* matrix metalloproteinase homologues), numbering them in the order we discovered them on the data base. Hence, At1-MMP (GenBank™ accession number Z97344, chromosome 4); At2-MMP (accession number AC002062, chromosome 1); At3-MMP, (accession number AC002396, chromosome 1); At4-MMP, (accession number AF062640, cDNA or AC007659, chromosome 2); At5-MMP, (accession number AC002062, chromosome 1); At3-MMP, (accession number AC002396, chromosome 2). Unlike any known MMP gene, the genomic sequences are contiguous, with no introns separating coding sequences. All sequences code for a signal peptide, a propeptide domain, and a catalytic domain; this domain structure is similar to MMP-7, with all other known vertebrate MMPs having at least one further domain, numbered in the order we discovered them on the data base. Hence, At1-MMP (GenBank™ accession number Z97344, chromosome 4); At2-MMP (accession number AC002062, chromosome 1); At3-MMP, (accession number AC002396, chromosome 1); At4-MMP, (accession number AF062640, cDNA or AC007659, chromosome 2); At5-MMP, (accession number AC002062, chromosome 1). The enzyme was incubated with MBP for 20 h at 37 °C, and products were analyzed by SDS-PAGE on a 10–20% gradient gel.

**N-terminal Amino Acid Sequencing—**N-terminal sequence determination of At1-MMP was performed by automated Edman degradation using a PE Biosystems Procise 491 protein sequencer following SDS-PAGE and transfer to polyvinylidene difluoride membrane (Millipore).

**Computer Software—**PCR primer design was performed using PRIME, GCG (Wisconsin); alignments were made using BESTFIT or PILEUP, GCG (Wisconsin); protein sorting signal analysis was performed using PSORT (University of Osaka, available on the Internet).
MMPs from A. thaliana

Expression of At1- to At5-MMP in Plant Tissue—Expression of the At-MMPs in flower (F), leaf (L), root (R), and stem (S) of 14-day-old A. thaliana was investigated using semiquantitative RT-PCR with PCR primers shown in Table I; the UBQ5 gene was used as a loading control. In all cases, samples from PCR were collected across a range of cycle numbers to ensure linearity of response. Fig. 2 shows representative experiments at 30 cycles for At-MMPs and 20 cycles for UBQ5. Although RT-PCR was able to detect transcripts for At1- to At5-MMP in all of the tissues examined, each At-MMP had a distinct pattern of expression, indicating a functionally different role for each. For At1-MMP, the expression level was F ≈ R ⊂ S ⊃ L; for At2-MMP, R ⊃ F > L > S; for At3-MMP, L ⊃ R ≈ F > S; for At4-MMP, F ⊃ S > L ⊃ R; and for At5-MMP, L ⊃ R > S > F. These expression patterns are all different from the Gm1-MMP in soybean, in which expression was only seen in leaves from approximately 10 days after leaf emergence until leaves became senescent, and Gm1-MMP was absent from stem and root tissue (6).

Expression, Purification, and Processing of At1-MMP in E. coli—The coding region of proAt1-MMP was amplified using RT-PCR as detailed under “Experimental Procedures.” The N terminus of the proenzyme was chosen on the basis of PSORT identification of the signal peptide to start at Ala-28. The C terminus of the wild-type enzyme contains a putative transmembrane domain, and this may suggest that further At-MMPs demonstrate that plant MMPs are most closely related to those from other invertebrates (sea urchin and nematode worm).

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TABLE II

Alignment of amino acid sequences

| Substrate | At1-MMP | At2-MMP | At3-MMP | At4-MMP | At5-MMP | Gm-MMP | hMMP7 |
|-----------|---------|---------|---------|---------|---------|--------|-------|
| At1-MMP   | 100 (100) | 50 (57) | 49 (55) | 59 (66) | 45 (55) | 48 (55) | 43 (49) |
| At2-MMP   | 50 (57) | 100 (100) | 73 (78) | 47 (55) | 59 (68) | 50 (55) | 40 (45) |
| At3-MMP   | 49 (55) | 73 (78) | 100 (100) | 52 (57) | 61 (68) | 48 (55) | 41 (47) |
| At4-MMP   | 59 (66) | 47 (55) | 52 (57) | 100 (100) | 46 (55) | 48 (55) | 40 (47) |
| At5-MMP   | 45 (55) | 59 (68) | 61 (68) | 100 (100) | 46 (55) | 47 (52) | 41 (47) |
| Gm-MMP    | 48 (55) | 50 (55) | 48 (55) | 48 (55) | 47 (52) | 100 (100) | 38 (45) |
| hMMP7     | 43 (49) | 40 (45) | 41 (47) | 40 (47) | 41 (47) | 38 (45) | 100 (100) |

Amino acid sequences were aligned pairwise using BESTFIT. Percentage of identity is shown, followed by percentage of similarity in parentheses.

Fig. 2. Expression of At-MMPs in four plant tissues. RT-PCR was performed on RNA from flower (F), leaf (L), root (R), and stem (S) of 2-week-old A. thaliana plants using primers as shown in Table I. Samples from the PCR were taken across a range of cycle numbers to ensure linearity of response; data here are from cycle 30 for At1- to At5-MMP primers and cycle 20 for UBQ5 housekeeping gene. Products were separated on a 2% agarose gel in 1 x TBE.

Fig. 3. SDS-PAGE of purified recombinant At1-MMP and processing by APMA and trypsin treatment. Recombinant At1-MMP (lanes 1 and 9) was treated with 1 mM APMA for 5 (lane 2), 15 (lane 3), 30 (lane 4), 60 (lane 5), 120 (lane 6), 180 (lane 7), or 240 (lane 8) min or with 1 µg/ml trypsin for 5 (lane 10), 15 (lane 11), 30 (lane 12), 60 (lane 13), or 120 (lane 14) min. Molecular weight markers (MW) (in thousands) are indicated on the left.

Fig. 4. N-terminal sequence of APMA- and trypsin-processed At1-MMP. Cleavage points for processing by trypsin or APMA treatment of At1-MMP are indicated by arrows. APMA treatment produced a major product with an N terminus at Ile-112 and a minor product with an N terminus at Thr-119.

Fig. 5. Degradation of myelin basic protein by At1-MMP and human MMP-3. Bovine MBP at 0.25 mg/ml and enzyme were incubated at 37 °C for 20 h, and the reaction products were analyzed by SDS-PAGE on a 10–20% gradient gel. Lane 1, 320 nM MMP-3 plus 75 µM BB-94; lane 2, 8 nM MMP-3; lane 3, 32 nM MMP-3; lane 4, 80 nM MMP-3; lane 5, 250 nM At1-MMP plus 75 µM BB-94; lane 6, 25 nM At1-MMP; lane 7, 50 nM At1-MMP. Molecular weight markers (MW) (in thousands) are indicated on the left.

TABLE III

Comparison of activity of At1-MMP against fluorogenic peptide substrates

| Substrate | kcat/Km (m^{-1} s^{-1}) |
|-----------|------------------------|
| Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 | 3,850 ± 150 |
| Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH2 | 17,550 ± 950 |
| Mca-Pro-Cha-Gly-Nva-His-Dpa-NH2 | 17,500 ± 500 |

Purified recombinant At1-MMP was incubated with three synthetic quenched peptide substrates across a range of [S] at fixed [E] at 37 °C to allow determination of kcat/Km. Errors are S.E. from two experiments.
The cell wall (and its remodeling) is crucial to growth and development of the plant, along with its response to environmental stresses and attack by pathogens or insects (14). It is thus tempting to speculate that the At-MMPs are involved in the remodeling of plant extracellular matrix in any or all of these situations.

Inhibition of At1-MMP—Using McaPChaGNvaHADpaNH₂ as substrate, the inhibition of At1-MMP by a peptide hydroxamate inhibitor (BB-94) and human TIMP-1 and TIMP-2 was examined. Activity was measured at fixed [S] over a range of inhibitor concentrations to calculate an apparent $K_i$ for each inhibitor by fitting the data to the tight binding inhibition equation of Morrison and Walsh (15). Although the data fit the equation well, this methodology only yields approximate values for $K_i$. Human TIMP-1 and TIMP-2 were both able to inhibit At1-MMP with apparent $K_i$ in the low nanomolar range, whereas BB-94, a broad spectrum inhibitor of matrix metalloproteinases, was approximately 10-fold more potent. The main, but four are likely to be anchored in the plasma membrane. At1-MMP is a functionally active enzyme, able to cleave both protein and peptide substrates and inhibited by mammalian TIMPs. The function of the At-MMPs in vivo is unknown, but we can speculate that they have roles in events in which the plant extracellular matrix is remodeled or broken down. Our future work will explore both biochemistry and function of the At-MMPs in detail.

**Acknowledgments**—This work would not have been possible without help and advice from Greg Dean, Vera Knäuper, Augustin Amour, Mike Hutton, Marc Laffleur, Ann Merryweather, and Nicola Williamson. N-termi nus sequencing was performed by Mike Valdres at the John Innes Centre Protein Sequencing and Peptide Synthesis Unit. BB-94 was a kind gift from British Biotech Pharmaceuticals Ltd. (Oxford, United Kingdom).

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J. Biol. Chem. 1999, 274:34706-34710.
doi: 10.1074/jbc.274.49.34706

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