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Short communication

Novel matrix metalloproteinase from the cyst nematodes *Heterodera glycines* and *Globodera rostochiensis*☆

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One approach to the development of control strategies for nematodes is the disruption of hatching. In some species, a cation-mediated change in eggshell permeability is an early indicator of hatching [1]. Additionally, numerous studies have indicated involvement in hatching of enzymes, particularly metalloenzymes. For example, the microbivorous nematode *Caenorhabditis elegans* secretes HCH-1 metalloproteinase into the perivitelline fluid before hatching [2], and a Zn^{2+}-dependent leucine aminopeptidase occurs in egg homogenates of the soybean cyst nematode, *Heterodera glycines* [3]. In distant invertebrate and vertebrate species, hatching enzymes (HE) belong to a prodigious family of Zn^{2+}-dependent metalloenzymes called matrixins or matrix metalloproteinases (MMPs) [4–9]. These enzymes regulate the composition and integrity of the extracellular matrix, an aggregate of collagens, elastins, glycoproteins and other macromolecules. Despite the critical importance of MMPs in other organisms for vital developmental processes such as eclosion and molting, the only known parasitic nematode MMP gene sequence is from *Gnathostoma spinigerum* [10]. In this paper, we report cDNA and genomic sequences encoding a novel MMP-like protease from the soybean cyst nematode *H. glycines* and a related sequence from the potato cyst nematode *Globodera rostochiensis*, the first MMP genes described in plant-parasitic nematodes.

Our main goal was to search for *H. glycines* genes encoding aminopeptidase-like enzymes similar to *C. elegans* aminopeptidase-1 [11]. Degenerate primers designed on the basis of this sequence (accession number NP502335) amplified PCR-products from both *C. elegans* and *H. glycines* cDNA; the latter was then labeled and used for screening the cDNA library as previously described [12]. One of nine positive clones contained a 2000-bp long insert with an open reading frame encoding a putative MMP-like protease. This was not surprising, since the probe contained a zinc-binding motif shared by aminopeptidases, MMPs and other zinc peptidases [13]. Although MMP was not the primary target of the screening, the isolated clone of this important protease was studied further.

The full-length cDNA sequence of the clone isolated from the cDNA library of *H. glycines* encodes a 586-amino acid protein we named Hg-MMP. The established sequence of the clone was used to design primers for the catalytic domain (sense primer, 5′-GGAACGTTCGACATTCT-3′; antisense, 5′-ACATCACTGAATGTTCG-3′), to confirm the Hg-MMP sequence in cDNA prepared from mixed-age *H. glycines* samples and genomic DNA extracted from fresh second-stage juveniles (J2) with the MasterPureTM DNA purification kit (Epicentre). The derived genomic sequence spanned 19 introns, with a total intron length of 3680 bp and the longest one comprising 1072 bp; intron #6 had a non-canonical splicing site GC-AG; exon #8 consisted of only 21 bp. The large number and length of Hg-MMP introns are highly atypical for nematode genes [14]. The antisense primer (5′-CGCCGTATGAGTCTGTG-3′) for Real-Time PCR (SYBR Green) was located on an exon-exon junction to avoid alteration of expression data by DNA contamination of samples; the sense primer was 5′-CTGGAGACCAACAATGC-3′. Relative transcriptional

Abbreviations: EST, expressed sequence tag; HE, hatching enzyme; Hg-MMP, heterodera glycines matrix metalloproteinase; J2, second-stage juveniles; MMP, matrix metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends

☆ Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. Nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers AY426765 through AY426767.

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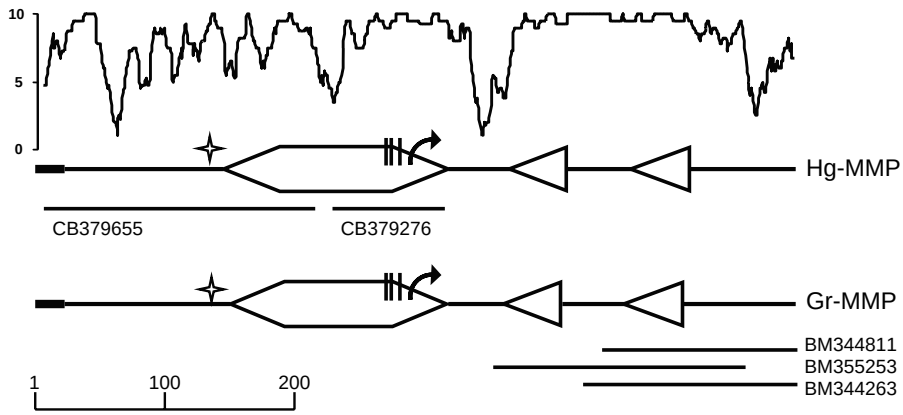

Fig. 1. Schematic diagram showing the structural organization of the putative matrix metalloproteinase (MMP) from *Heterodera glycines* and *Globodera rostochiensis*. The lengths of the geometrical figures are proportional to sequence length. The signal peptide is represented by a thick horizontal bar; the cysteine switch, by a star; the zinc-binding region with the zinc ligands and the methionine-turn, by the hexagon with vertical bars and an arrow, respectively; and the hemopexin repeats, by the triangles. Amino acid homology between Hg-MMP and Gr-MMP (upper curve) was measured for blocks of 10 residues. EST database fragments with 99–100% identity to the MMP illustrated above them are shown by horizontal bars with accession numbers.

levels were monitored during development; the *H. glycines* actin sequence previously established in our laboratory (accession no. AF318603) was used as a reference gene and the $2^{-\Delta\Delta CT}$ formula was used for quantification (PE Applied Biosystems, Foster City, CA, P/N 4303859). During egg development, the normalized level of Hg-MMP RNA rose from 1.0 (egg samples with mostly embryos) through 1.8 (developmentally intermediate sample) and peaked at 5.2 (eggs primarily containing fully developed J2). After hatching, Hg-MMP transcript levels returned to near pre-hatch levels: expression ratios measured 1.6 for hatched J2 and 1.8 for females. The pattern found in these preliminary experiments concurs with an expression pattern expected for genes involved in embryonic development and hatching.

The deduced amino acid sequence of Hg-MMP indicated that the 65.9-kDa protein is a pre-proenzyme consisting of signal peptide, propeptide with activation locus, catalytic domain with a zinc-binding site, and C-terminal hemopexin domain (Fig. 1). A similar domain arrangement is displayed by many MMPs [15]. The 22-amino acid long signal peptide in Hg-MMP indicates that this enzyme should be a secretory protein. The highly conserved sequence PRCGXXD within the propeptide (the first matrixin signature) is characteristic for the “cysteine switch” mechanism of activation of MMPs [16]. Hg-MMP has (at position 132–138) a rather divergent sequence for this motif, KRCANSD, with C-134 conceivably involved in maintaining latency of the enzyme. Similarly to plant MMPs, Hg-MMP lacks a proprotein-processing furin recognition sequence downstream of the cysteine switch.

The putative catalytic domain of about 170 residues in Hg-MMP (Fig. 2) includes the zinc binding motif HEIGHALGLRH (complying with the consensus motif HEXGHXXGXXHS known for approximately 1300 other metalloproteinases), containing the three His residues (H-271, H-275 and H-281) involved in coordination of the catalytic zinc atom at the active site, the catalytic Glu (E-272) residue, and the Ser residue (S-282) that distinguishes MMPs from other metalloproteinases. The catalytic domain has a Met residue, known as the Met-turn, seven residues downstream of the zinc-binding site, conserved in all MMPs and proposed to play a crucial role in the structure of these enzymes [17]. Remarkable characteristics of Hg-MMP include the absence of the third matrixin signature—the binding region for a second zinc atom (also known as the structural zinc) and a calcium ion [18]. In the Hg-MMP sequence this signature lacks all three His residues (e.g., H-203, H-218 and H-228 in soybean) thought to be essential for zinc binding and is substituted by an inserted mixed charge segment with the pocket at 218 to 219: 209-DGAKKEKSK|NG|EEEKEKEGK. Finally, the deduced sequence contains a C-terminal fragment of about 200 residues with sequence similarity to hemopexin (two repeats); most MMPs usually contain three or four repeats. A hinge-region is located between the catalytic and hemopexin-like domains and is proline-rich.

Analysis of the deduced amino acid sequence of Hg-MMP did not allow us to assign it to any of the four main MMP subfamilies (the collagenases, gelatinases, stromelysin and membrane type- or MT-MMPs). The lack of the fibronectin-like domain present in gelatinases, the transmembrane domain possessed by MT-MMPs, the insertion of mostly hydrophobic residues in the C-terminus of the catalytic domain of stromelysins, along with other basic characteristics, exclude Hg-MMP from those subgroups. Despite the strongest similarity to collagenases, Hg-MMP lacks two of the three essential residues (Tyr, Asp, and Gly) that are conserved in all collagenases and are proposed to be essential determinants of their specificity [19]. The topographically equivalent residues in Hg-MMP are Ile-263,

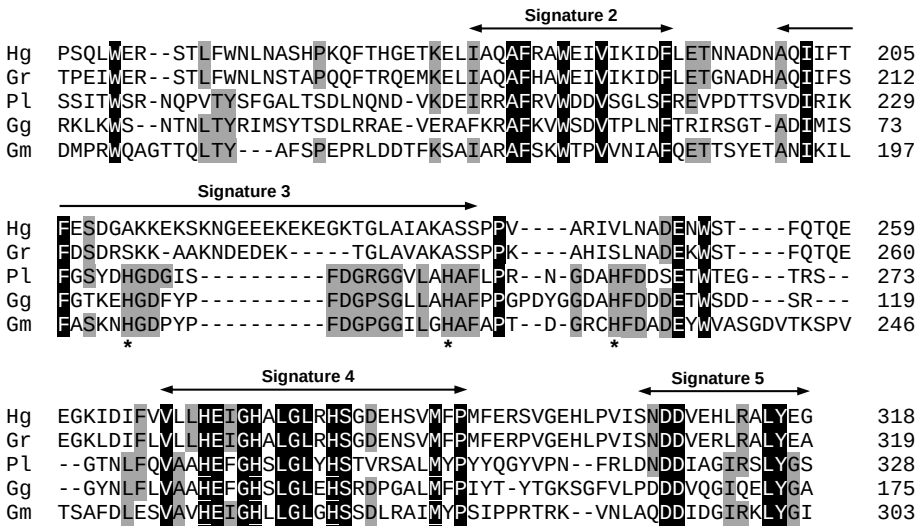

Fig. 2. Amino acid sequence alignment of the catalytic domain of MMPs from selected species. Four matrixin signatures (2–5) are designated by arrows; signature 3 is absent in cyst nematode MMPs. Conserved histidine residues involved in zinc-binding and the conserved methionine residue (the Met-turn) are underlined. Amino acids identical in all sequences are shaded black; residues identical in some but not all species are shaded grey. Histidine residues purportedly involved in binding of structural zinc and absent from the nematode sequences are marked with asterisks. Hg, *Heterodera glycines* MMP; Gr, *Globodera rostochiensis* MMP; Pl, hatching enzyme from the sea urchin *Paracentrotus lividus* (P22757); Gg, matrix metalloproteinase-13 from chicken, *Gallus gallus* (AAC34604); Gm, metalloendoproteinase-1 precursor (SMEP1) from soybean, *Glycine max* (P29136).

Asp-284 and His-286. Therefore, we suggest that Hg-MMP should be included in a growing category of "other MMPs".

Using PCR with primers based on the Hg-MMP sequence (sense, 5′-TCGACTTTCTGGAGACC-3′; antisense, 5′-TTGGGCCGTTTTCTGATC-3′), we amplified a fragment of an Hg-MMP homologue from *G. rostochiensis* approximately 700 bp long. The established sequence was used further to design primers for PCR and the RACE method, thereby resulting in construction of the complete coding sequence of an MMP-like gene from *G. rostochiensis* (Gr-MMP). The deduced proteins have identical architecture and very high amino acid and nucleotide similarities (78 and 77%, respectively). Interestingly, Gr-MMP shared the unusual characteristics of Hg-MMP—the diverse sequence for the Cys-switch, the lack of the three His residues essential for the binding of the structural zinc atom, and the inserted mixed charge segment. Homology of 90–100% exists at functionally valuable areas: e.g., the catalytic domain (with the exception of insert sequence) and the hemopexin domain (Fig. 2). In contrast, the propeptide, hinge-region and C-terminus have lower similarity, in some parts below 50%. The homology between MMP amino acid sequences from *H. glycines* and from *G. rostochiensis* is strikingly high, thereby indicating that the novel MMP from both species represents the same type of enzyme.

When compared to all available full-length proteins, the entire Hg-MMP amino acid sequence exhibited greatest homology to the *C. elegans* matrixin NP_506678 (23% amino acid identity). Among numerous MMP-like genes described for *C. elegans*, only four, including NP_506678, have a domain arrangement similar to Hg-MMP. The MMP-like gene from the animal-parasitic nematode *G. spinigerum* does not have a domain architecture identical to Hg-MMP [10]. When only the catalytic domains are compared, Hg-MMP exhibits greater similarity to an MMP-like sequence from the soybean plant (*Glycine max*) and HE from the sea urchin *Paracentrotus lividus* (21–23% amino acid identity) than to nematode sequences.

When the nucleic acid sequence of Hg-MMP was compared to publicly available nematode sequences, two matches to reported *H. glycines* sequences were revealed (Fig. 1). We also discovered the existence of EST fragments from *G. rostochiensis* with 99–100% nucleic acid identity to Gr-MMP (Fig. 1), and from *G. pallida* (BM416460, BM416273) with 89–92% identity to Gr-MMP. Much lower similarity was found between Hg- and Gr-MMPs and EST sequences from the plant-parasitic nematodes *Meloidogyne incognita* (AW571335, AW828537) and *M. javanica* (BI143144) and from the animal parasites *Ostertagia ostertagi* (BM897604), *Ascaris suum* (CA303636) and *Brugia malayi* (AA842852). The distinctly lower level of homology between the cyst nematode sequences and the non-cyst nematode sequences suggests that cyst nematode MMPs may be unique in both structure and function.

Metalloproteinases are the most diverse of the four main types of protease, with more than 30 families identified to date [20]. The reported sequences, the first full-length metalloproteinase genes from plant-parasitic nematodes, expand our knowledge of these proteases. The poor homology of Hg-MMP with any protein from any organism with a completely sequenced genome is quite remarkable. In light of the role of MMPs in hatching in other organisms, further investigation of MMP function in cyst nematodes is warranted.

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