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Cloning of a salivary gland metalloprotease and characterization of gelatinase and fibrin(ogen)lytic activities in the saliva of the Lyme Disease tick vector *Ixodes scapularis*

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Abstract

The full-length sequence of tick salivary gland cDNA coding for a protein similar to metalloproteases (MP) of the reprolysin family is reported. The *Ixodes scapularis* MP is a 488 aminoacid (aa) protein containing pre- and pro-enzyme domains, the zinc-binding motif HExxHxxGxxH common to metalloproteases and a cysteine-rich region. In addition, the predicted amino-terminal sequences of *I. scapularis* MPs were found by Edman degradation of PVDF-transferred SDS/PAGE-separated tick saliva proteins, indicating these putative enzymes are secreted. Furthermore, saliva has a metal-dependent proteolytic activity towards gelatin, fibrin(ogen) and fibronectin, but not collagen or laminin. Accordingly, *I. scapularis* saliva has a rather specific metalloprotease similar to the hemorrhagic proteases of snake venoms. This is the first description of such activity in tick saliva and its role in tick feeding and *Borrelia* transmission are discussed.

Keywords

Ixodes scapularis; *Borrelia burgdorferi*; tick; hematophagy; collagen; fibrinogen; fibrino(gen)olytic; ADAM; reprolysin; blood-sucking

Introduction

Ticks feed exclusively on blood throughout their lifetimes. After mechanically attaching to their vertebrate host with their mouthpart, they initially inject gluey saliva that helps to cement the ticks to their hosts; then, for several days, they alternately suck blood and salivate into their hosts [1]. The tick feeding lesion on a non-immune host is characterized by a cavity filled with blood. Remarkably, blood does not clot into this cavity and no host repair to this injury occurs during feeding, although the tissue surrounding this cavity usually contains an infiltrate of neutrophils and monocytes [2]. It has been proposed that tick saliva is essential for maintaining the feeding cavity by being a rich source of antihemostatic, antiinflammatory, and immunosuppressive compounds [3–6].

Currently known pharmacologically active compounds found in *Ixodes scapularis* saliva include prostaglandin E_2 [7], Isac, an inhibitor of the alternative pathway of complement [8] and Ixolaris, an inhibitor of the extrinsic pathway of blood coagulation [9]. Additionally, an

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uncharacterized protein binds to and inhibits IL-2 [10], a salivary apyrase destroys the platelet agonist ADP [7], a dipeptidyl peptidase destroys the pain-producing peptide bradykinin [11], and this or another peptidase destroys anaphylatoxins [12]. Neutrophil aggregation and granule release is also inhibited by this tick saliva [13], as are platelet aggregation by collagen and platelet- activating factor (PAF) [7]. It is thus apparent that tick saliva is a complex mixture of potent pharmacologically active compounds.

To gain insight into the complexity of *I. scapularis* salivary compounds, we randomly sequenced clones in a salivary gland cDNA library from this tick and described the coding region for 87 such clones that are possibly associated to secreted products [14]. Among these sequences, we found two that coded for metalloproteases similar to atrolysins, which are proteases found in snake venom that have fibrinogenolytic and gelatinase activities [17]. Furthermore, the predicted aminoterminal sequence of the active enzymes were found through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separating proteins from tick saliva [14], indicating that these putative metalloproteinases were secreted. Accordingly, we tested whether the enzymatic activity of *I. scapularis* saliva was consistent with those indicated by the two cDNA clones having sequence similarity to snake metalloproteases. We report this salivary activity for the first time in hard ticks.

Material and methods

Water and organic compounds

All water used was of 18 MΩ quality produced by a MilliQ apparatus (Millipore, Bedford, MA). Organic compounds were obtained from Sigma Chemical Corporation (St. Louis, MO) or as stated.

Ticks and tick saliva

Tick saliva was obtained by inducing partially engorged adult female *I. scapularis* to salivate (3–4 days post attachment to a rabbit) into capillary tubes using the modified pilocarpine induction method [8]. Saliva samples were stored frozen at −75°C until needed.

Total RNA extraction

Five pairs of salivary gland from ticks feeding for 3 days were used for total RNA isolation using TRIzol reagent (Gibco-BRL, NY). In an Eppendorf tube, 1 ml TRIzol was added to the glands and homogenized. Two hundred µl of chloroform was added and the tube vigorously shaken for 15 sec, and centrifuged at 10,000 g for 10 min at 2–8 °C. The upper aqueous phase was transferred and 500 µl of isopropyl alcohol was added followed by sample mixing by repeated inversions. RNA was finally re-suspended in 5 µl of water at a final concentration of 400 ng/µl (2 µg total).

Reverse transcription and amplification of 5`- cDNA ends

This was performed using the GeneRacer Kit (Invitrogen, CA) following manufacturer instructions. Briefly, GeneRacer Oligo dT primer (5`-CTGTCAACGATACGCTACGTAACGGCCATGACAGTG$(T)_{18}$ was added to 2 µg total RNA (in 10 µl) followed by addition of Cloned Avian Myeloblastosis Virus (AMV) Reverse Trancriptase for 1 hour at 45°C. For 5`-cDNA amplification, the forward primer 5`-ATG, TCG, GGA, CTC, AGC, CTG, AAA, TTG, TGG, AT – 3 was designed based on the full length clone reported for a cDNA coding for a metalloprotease from *Ixodes ricinus*. The reverse primer (GeneRacer 3` primer) was 5`- GCT, GTC, AAC, GAT, ACG, CTA, CGT, AAC, G – 3`. Touchdown PCR was performed using Platinum *Taq* polymerase (Gibco-BRL). Condition were as follows: 94°C for 2 min, 5 cycles of 94°C for 30 sec, 72°C for 1 min; 5 cycles of 94°

C for 30 sec, 70°C for 1 min, 25 cycles of 94°C at 30 sec, 62°C at 30 sec, 68°C for 1 min, and 1 cycle of 68°C for 10 min. PCR products were visualized on a 1.1% agarose gel with ethidium bromide.

Cloning I. scapularis metalloprotease

I. scapularis metalloprotease was cloned in TOPO pCR4 vector (Invitrogen, CA) following manufacturer`s instructions. TOP10 chemically competent cells were transformed by heat shock procedure and plated in ampicilin-agar plates overnight. Colonies were picked and used for cyclo-sequencing reactions using the DTCS labeling ki (Beckman Coulter Inc., CA). Samples were sequenced on a CEQ Beckman 2000 (as described [15] using M13 forward and M13 reverse universal primers. Internal sequences were amplified using five gene-specific forward primers and M13 reverse primer.

Gelatinase assays

Gelatinase assays were conducted in 50 µl of medium containing 25 mM Hepes buffer, pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$, 0.1 mg/ml fluoresceinated gelatin (Molecular Probes, Eugene, OR), and the indicated amounts of tick saliva. Fluorescence (excitation filter = 490 nm, emission filter = 530 nm) was read at 1 to 3-min intervals in a fluorescence plate reader (Fluorolite 1000; Dynex Technologies, Chantilly, VA) using black U-shaped 96-well plates from the same manufacturer.

SDS-PAGE-based protelolytic experiments

Human fibrinogen (FNG) was dissolved to 2.5 mg/ml in 50 mM Hepes, pH 7.4, containing 150 mM NaCl and either 1 mM $CaCl_2$ or 1 mM EDTA. Fifteen µl of these solutions were mixed with 5 µl of tick saliva (previously incubated for 90 min at 37°C with 1 µl of NaCl 0.15 M or 1 µl 50 mM EDTA). At the indicated times, 3-µl aliquots were removed, mixed with sample buffer containing dithiotreitol (Invitrogen, San Diego, CA), and heated at 70°C before being applied to gels. Plasma fibronectin (FN) (CalBiochem, San Diego, CA) was diluted to 1 mg protein/ml in the Hepes buffer with either calcium or EDTA, as above. Type I collagen (COL-I) was first disolved to 5 mg/ml in 100 mM Na acetate pH 4.0, before 10-fold dilution to the substrate Hepes buffers, thus giving a final concentration of 0.5 mg/ml. FN, COL-I, COL-IV and laminin samples were treated as for the FNG experiment. NuPAGE 4–12 % gradient gels, 1 mm thick (Invitrogen), using reducing MES buffer were run according to manufacturer’s recommendations to separate the indicated proteins treated with tick saliva. To estimate the mol wt of samples, SeeBlue™ markers from Invitrogen (myosin, BSA, glutamic dehydrogenase, alcohol dehydrogenase, carbonic anhydrase, myoglobin, lysozyme, aprotinin, and insulin chain-B; all from Invitrogen) were used.

Assay of fibrinolytic activity

Human FNG (2 mg/ml) in Hepes 50 mM, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$ was mixed with thrombin to give 0.5 U enzyme/ml and the solution quickly added to a plastic 2-cm-diameter well of a 12-well plate. After 60 min at 37°C, 10 µl of tick saliva was added to the surface of the clot and the plate incubated in a humid atmosphere for 12 h at 37°C, when it was stained with Coomassie blue.

Assay of clotting activity

To detect possible FNG clotting by tick saliva, 15 µl of a solution containing 2 mg/ml of FNG in Hepes 50 Mm, pH 7.4, 100 mM NaCl, and 5 mM $CaCl_2$ were mixed with 5 µl of tick saliva and the vial kept at 37°C. At 3-min intervals, the vial was shaken by hand to determine whether the solution had gelled.

Sequence analyses

Alignments and phylogenetic trees were made with the ClustalX program [16].

Results and discussion

We have previously reported [14] the partial sequence of two cDNA clones, found in a salivary gland library of the tick *Ixodes scapularis*, which have similarity to hemorrhagic snake venom metalloproteases (MP) of the reprolysin family [17,18]. In an attempt to obtain the full-length sequence of *Ixodes scapularis* MP, herein called *I. scapularis* MP1 we have isolated total RNA from the salivary gland from ticks after 3 days of blood feeding. cDNA was obtained by reverse transcription as described in Material and Methods section. Amplification of gene-specific *I. scapularis* MP1 was performed by touchdown PCR using a forward primer designed based on the starting codon reported for the homologous full-length clone of *I. ricinus* MP (e value=$1e^{-148}$, gi 5911708) and GeneRacer 3` reverse primer. Visualization of PCR products shows that only one band of approximately 1.7 kb was obtained (not shown) and an aliquot was used for cloning *I. scapularis* MP1 in pCR4-TOPO vector and transformation of TOP10 *E. coli* chemically competent cells. Positive clones were amplified and sequenced with M13 forward and reverse primers in addition to five internal gene-specific primers. The full-length cDNA and predicted amino acid sequences of *I. scapularis* MP1 is shown in Figure 1. According to the output of Signal P server and the N-terminus YKIP obtained by Edman degradation of salivary proteins [14], we conclude that *I. scapularis* MP1 contains a pre-enzyme domain (signal peptide) of 19 amino acids, a pro-enzyme domain of 150 amino acids, and a mature protein of 319 amino acids, with a theoretical mol wt 36937.79, and pI 8.66.

Figure 2A shows the alignment of the mature form of *I. scapularis* MP1 (gi AY264367) with two other *I. scapularis* [14] respectively called *I. scapularis* MP2 (gi 22164294) and *I. scapularis* MP-3 (gi 22164296), in addition to *I. ricinus* (gi 5911708) putative salivary metalloproteases. Close inspection of these sequences (Fig. 2A) reveals that they have the zinc binding (catalytic) domain of the reprolysins family (HELGHNLGxxHD) [17,18], except for one conserved G-> A substitution. The clan MB of metalloproteases contains the zinc-binding motif HExxHxxGxxH and a distally located methionine (usually 25 residues distal to the first H of the zinc-binding domain) [19]. Due to this conserved Met and the zinc-binding motif, this family is also known as the metzincins [20,21]. The four *Ixodes* sequences have the zinc-binding domain of metzincins and a methionine 36 residues distally located from the start of the zinc-binding domain, 11 residues more than found in clan MB.

Alignment of the tick sequences with four other reprolysins (Fig. 2B) shows the conserved Met among all sequences, allowing for two regions of insertions in the tick sequences which account for the 11 extra residues (Fig. 2B). These insertions are flanked by Gly and Pro residues (Fig. 2B), which are known to allow loops to form in the protein structure. The Met of the tick sequences is followed by Ser-Tyr, reminiscent of the His-Tyr of the astacin subfamily of the M12 family of metalloproteinases (EC 3.4.24.21) [19,22]. This suggests that the two *I. scapularis* sequences belong to the M12 family of metalloproteases [19,22].

Snake venom metalloproteases are organized into four classes [18]. The P-I class has only a protease domain and pre-pro sequences, and a molecular mass between 20 and 30 kDa. The P-II class has an additional disintegrin domain and a spacer region between the protease and disintegrin domains. P-II molecular mass is 30 to 60 kDa. The disintegrin domain most commonly contains the triad RGD flanked by cysteines; this loop inhibits the binding of FNG to integrins. The disintegrin domain has several additional conserved cysteine residues, with a total of six or more [23]. The P-III class has the domains noted above for the P-II class, followed by a high cysteine domain with a characteristic sequence pattern of 11 cysteines ($CX_6CX_4CX_6CX_{14}CX_{12}CX_9CX_6CX_4X_{4/6/10}CX_{15/18/19}CX_5CX_4C$)) [17,18]. They vary from

60 to 90 kDa. Finally, the P-IV class has all the domains of the P-III plus a lectin domain. Many P-IV class proteins do not display proteolytic activity and are thought to function as extracellular matrix components.

I. scapularis salivary metalloprotease sequences have mol wt compatible with the P-II class. They each have a cysteine-rich domain (10 cysteines) following the active site (Fig. 1 and 2), but no RGD triplet typical of the disintegrins. The tick cysteine residue spacing follows neither the pattern found in disintegrins [23], nor that found in the cysteine-rich domain of the P-III class of enzymes [17,18]. Although the function of the tick cysteine-rich domain is not known, it may provide binding sites to extracellular matrix proteins, as proposed for other metalloproteinases [24]. Binding of the tick metalloproteases to the host matrix would increase its efficiency in maintaining the feeding cavity, and avoid spread of the activity to the host's blood circulation.

Because the two tick protein sequences described above have similarity to metalloproteases with gelatinase activity, and because Edman degradation of protein bands separating *I. scapularis* saliva by SDS-PAGE revealed sequences predicted by the two clones, we investigated whether *I. scapularis* saliva was able to hydrolyze gelatin. Indeed, *I. scapularis* saliva digested gelatin, and the reaction was inhibited by the divalent cation chelator EDTA (Fig. 3A). Upon serial dilution, activity could be detected with as little as 0.03 μl of tick saliva per 50 μl of reaction mixture (results not shown). Of interest, the EDTA inhibition was time dependent, following an exponential decay function with a time constant of 38 ± 2.4 min (mean ± SE of nine determinations; r^2 of exponential regressions = 0.93 ± 0.01) (Fig. 3B). This slow enzyme inactivation may result from tight binding of the zinc activator to the enzyme.

To investigate the substrate specificity of *I. scapularis* proteolytic activity, we incubated tick saliva with FNG, FN, COL-I, COL-IV, and laminin and analyzed the resulting mixture by SDS-PAGE under reducing conditions. The Aα chain of FNG, but not the Bβ or γ chains, was completely hydrolyzed by tick saliva. Most of the Aα chain was hydrolyzed by 15 min of incubation (Fig. 4A). The proteolytic degradation specificity of the Aα chain of FNG is observed in several metalloproteases, including some snake venom metalloproteases [18,25], as well as in spider metalloproteases [26]. FN was also quickly degraded to a smaller fragment by *I. scapularis* saliva in the presence of Ca^{++} but not in the presence of EDTA (Fig. 4B). We found no evidence of hydrolysis of COL-I, COL-IV, or laminin when these were incubated with tick saliva (results not shown). Together, these experiments indicate that *I. scapularis* saliva has a rather specific metallopeptidase acting on FNG and related molecules.

Thrombin action on FNG cleaves a peptide from the amino terminal region of the Aα chain, exposing the fibrin polymerization site distal to the cleavage. *I. scapularis* saliva acts on the same chain, therefore we determined whether incubation of tick saliva with FNG could lead to transient formation of a clot or, alternatively, whether tick saliva could lead to dissolution of a fibrin clot. Close inspection of the mixture of saliva and FNG at 3-min intervals revealed that the solution was liquid for the first 15 min (results not shown), after which most of the Aα chain was hydrolyzed (Fig. 4A). On the other hand, incubation of tick saliva with a fibrin clot led to clot dissolution (Fig. 4C). We conclude that *I. scapularis* saliva has a fibrinolytic activity and that, while cleaving FNG, no fibrin clot is formed.

The role of salivary metalloproteinases in tick feeding appears to be related to its antifibrinogen- and antifibrin-specific activities which, similar to the hemorrhagic proteases of snake venom [17,18], should confer an additional antihemostatic activity to the tick's salivary antihemostatic cocktail [4,5]. In the context of *Borrelia* transmission by *I. scapularis*, it is interesting to speculate that tick saliva metalloproteinase may also modulate *Borrelia* transmission. In fact, it has been shown that *Borrelia* spirochetes upregulate release and activation of matrix

metalloproteinase gelatinase B (MMP-9) and collagenase 1 (MMP-1) in human cells, enhancing the penetration of *B. burgdorferi* across extracellular matrix components [27]. Prevention of fibrin clot formation by *I. scapularis* metalloproteases and other salivary antihemostatic agents [4,5] and dissolution of the fibrin clots eventually formed around the feeding cavity of *Ixodes* may similarly aid spirochete dissemination through vertebrate tissues.

Finally, it should be noted that although we found the gelatinase and fibrinogenolytic activities in *I. scapularis* saliva through salivary cDNA sequences coding for proteins suggesting these activities, we cannot presently verify that all activity reported in this paper derives solely from those three proteins. Their aminoterminal sequences were found in protein bands obtained by separation of salivary proteins by SDS-PAGE [14], making it very likely. Attempts to separate saliva under non-denaturing conditions using either molecular sieving or anion-exchange protocols recovered no gelatinase activity, indicating that the activities either bound irreversibly to the column or self inactivated during the purification procedure. In either case, this report represents, to our knowledge, the first description of a fibrinogenolytic activity in any hematophagous arthropod saliva, although this type of activity has been described in the salivary glands of leeches [28–30], and in brown spider venom [26]. It is possible that such activity is widespread in ticks and other blood-sucking animals.

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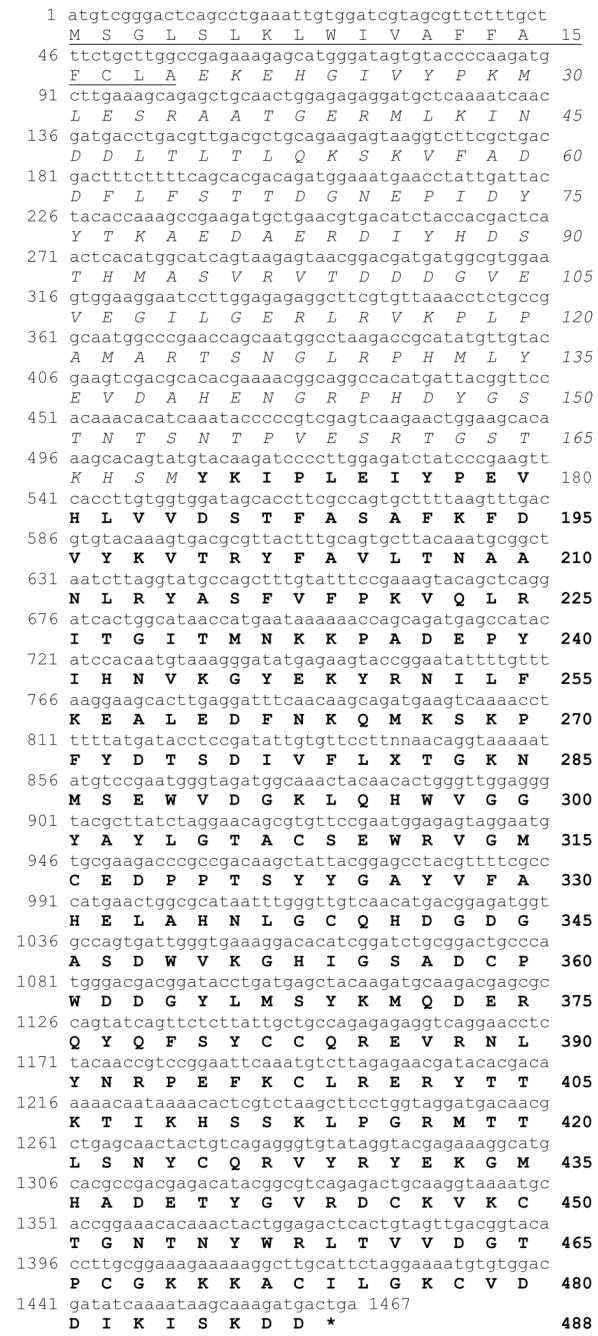

Fig. 1.

Full-length nucleotide sequence and deduced amino acid sequence of *Ixodes scapularis* metalloprotease (*I. scapularis* MP1)(gi AY264367). The nucleotides and aminoacids are numbered from the translation starting site ATG. The signal peptide sequence or pre-enzyme (1–19 aa) is in bold underlined. The pro-enzyme (20–169 aa) is in bold italicized. The mature protein (170–488 aa) starting with the sequence YKIPL obtained by Edman degradation of salivary proteins [14] is in bold.

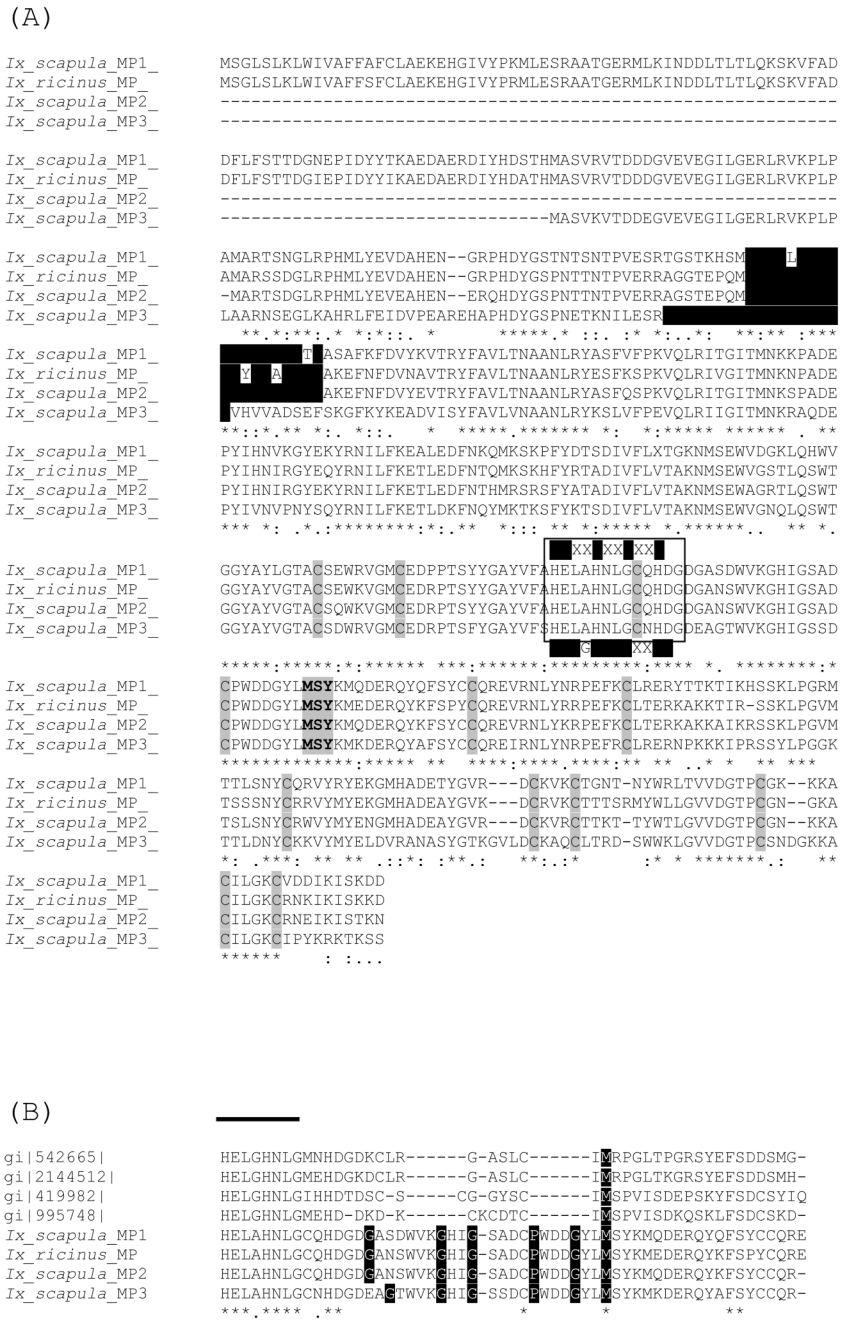

Fig. 2.

(A) Alignment of *I. scapularis* MP1 sequence (*Ix_scapula*_MP1; gi AY264367) having similarity to other *Ixodes* MPs: *I. scapularis* MP2 (*Ix_scapula*_MP2; gi 22164294), *I. scapularis* MP3 (*Ix_scapula*_MP3; gi 22164296), and *I. ricinus* MP (*Ix_ricinus*_MP; gi 5911708). Peptides YKIP… and AADT…(shown in reversed background) were found by Edman degradation of protein bands from SDS-PAGE gels separating tick saliva and indicate the N-terminus of the mature protein [14]. The box indicates, above the aligned sequences, the metzincin zinc-binding motif HEXXHXXGXXH [22] and, below the aligned sequences, the snake venom metalloprotease active site consensus sequence [17,18]. The reversed background residues in these motifs indicate identities in the tick sequences. The triad MSY following the

zinc-binding box is the putative methionine turn found after the zinc-binding domain [22]. All cysteines are shown in reversed background. (B) Alignment of *I. scapularis* putative reprolysins with atrolysins to show conservation of the carboxy terminal M (arrow) if two insertions flanked by glycine and proline residues (in reverse background) are considered. The line above the sequences indicates the zinc-binding domain of the active center. The symbols under the sequences indicate identity (*), highly conserved (:), and conserved (.) residues. The NCBI accession numbers of non-tick sequences are shown after the gi|.

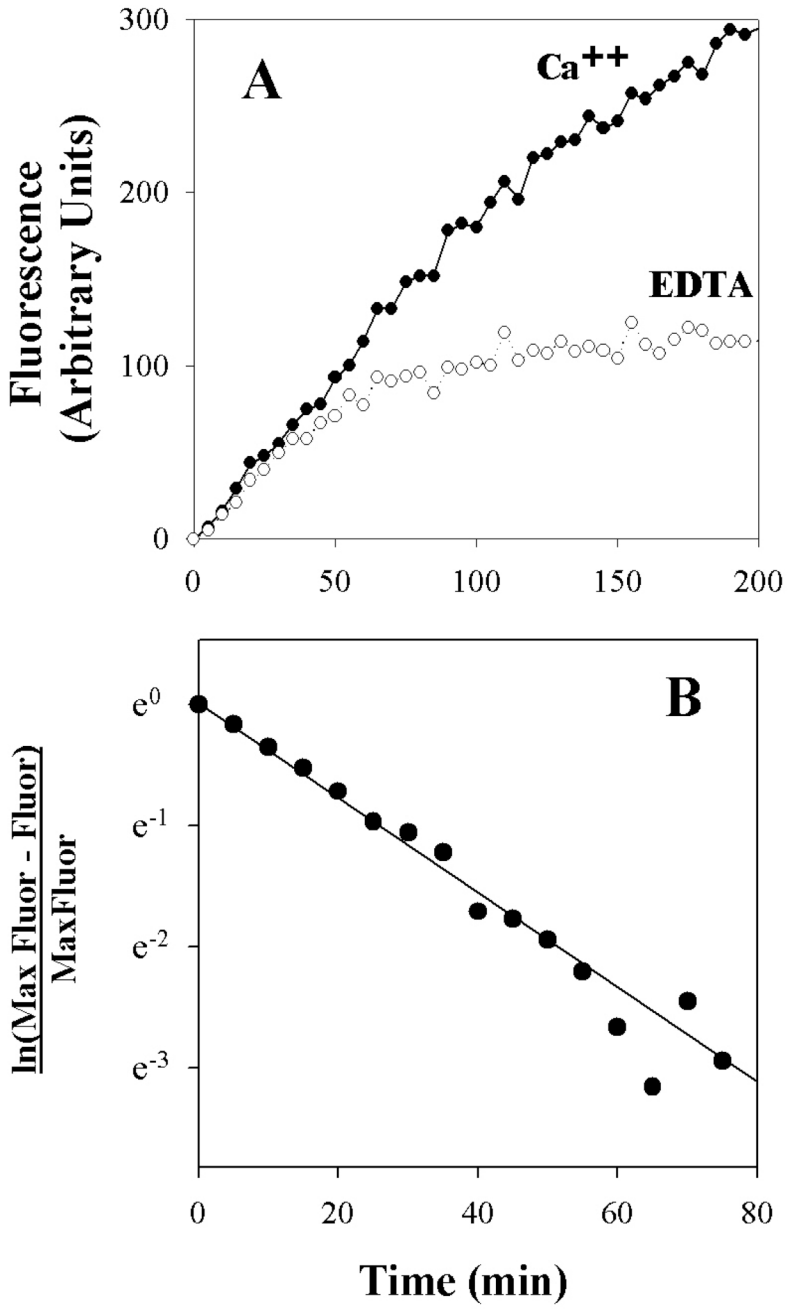

Fig. 3.

Gelatinase activity of *I. scapularis* saliva. (A) Graph represents increase in fluorescence of fluoresceinated gelatin (0.1 mg/ml in Hepes buffer 25 mM NaCl 150 mM containing either 5 mM $CaCl_2$ or 5 mM EDTA) following addition of diluted tick saliva to give a total amount of 0.125 μl in a 50-μl reaction mixture. Similar results were obtained with four other pools of tick saliva. (B) Exponential loss of activity of tick gelatinase activity in the presence of 5 mM EDTA. Maximal fluorescence (MaxFluor) was estimated as the average of the last five fluorescence level determinations in the presence of EDTA. Each time point was plotted in the natural logarithmic scale as the ratio of the difference between MaxFluor minus the

fluorescence at each time point (Fluor) divided by MaxFluor. Similar results were obtained with four other pools of saliva, each done at three different saliva dilutions.

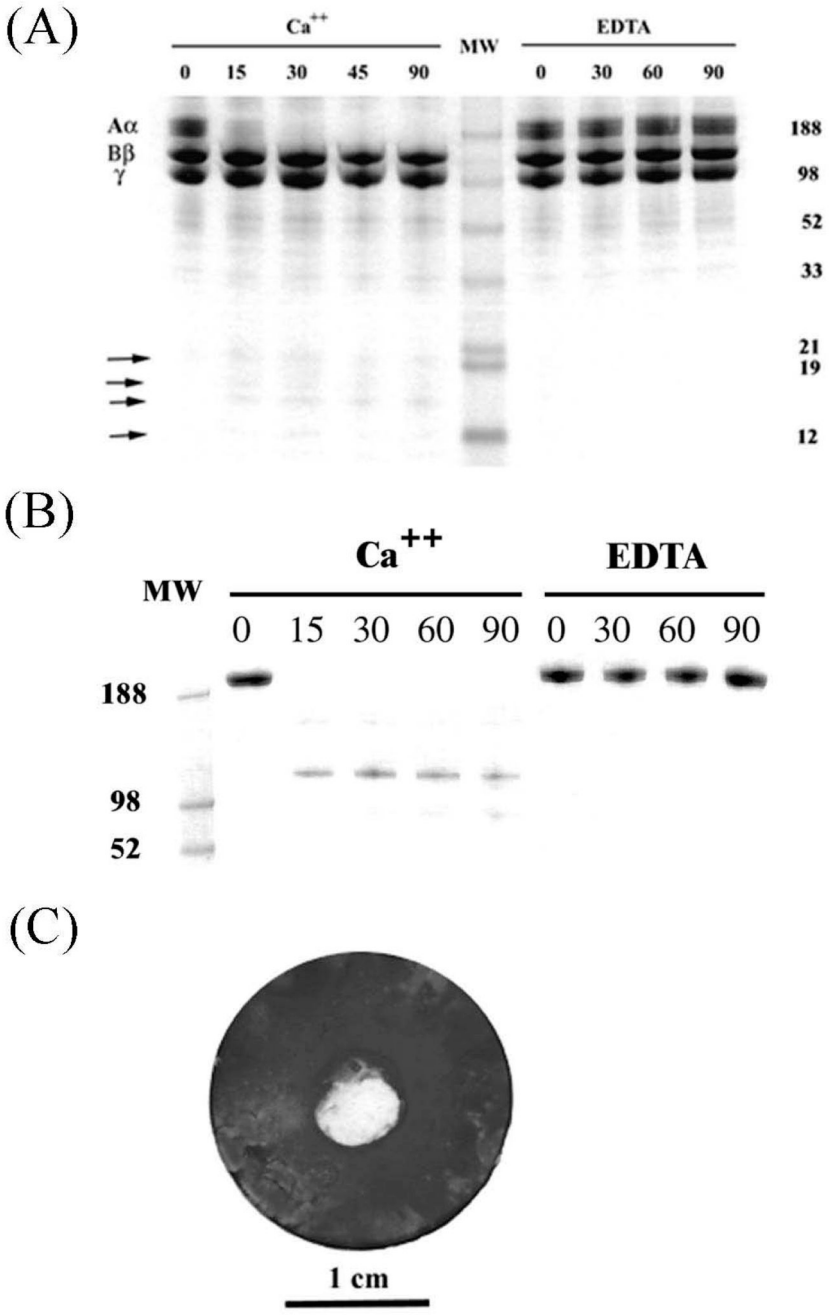

Fig. 4.

(A) Hydrolysis of the Aα peptide chain of FNG by *I. scapularis* saliva. FNG was incubated with tick saliva for the indicated times (in minutes) in the presence of $CaCl_2$ (5 mM) or EDTA (5 mM) before being heated at 70°C with sample buffer to terminate the reaction. Lane MW contains the mol wt markers, with their numbers indicated on the right. The Aα, Bβ, and γ chains of FNG are indicated. Arrows indicate degradation products observed when saliva was incubated with FNG in the presence of Ca^{++}. Two other experiments with two different saliva pools gave identical results. For more details, see Materials and methods. (B) Hydrolysis of FN by *I. scapularis* saliva. Fibronectin was incubated with tick saliva for the indicated times (in minutes) in the presence of $CaCl_2$ (5 mM) or EDTA (5 mM) before being heated at 70°C

with sample buffer to terminate the reaction. Lane MW contains the mol wt markers, with their numbers indicated on the left. Two other experiments with two different saliva pools gave identical results. For more details, see Materials and methods. (C) Fibrinolytic activity of *I. scapularis* saliva. The gel that has not been digested is Coomassie blue stained and shown in black in the Figure. The central digested gel is not stained and represents the fibrinolytic activity of saliva. For more details, see Materials and methods. Bar, 1 cm length.