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Purification of a serine protease and evidence for a protein C activator from the saliva of the tick, Ixodes scapularis

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Abstract

The saliva of ticks is critical to their survival as parasites and hematophagous animals. In this study, we have purified an enzyme with trypsin-like activity from the saliva of the tick vector of Lyme Disease, *Ixodes scapularis*. This enzyme, named as IXOSP (*Ixodes scapularis* salivary serine protease), is a 29.9 kDa molecule with N-terminus FPxMVxLRIKxR. A BLAST search identified IXOSP as a secreted serine protease (AAY66740) with a conserved catalytic triad His, Asp, and Ser. In vitro studies demonstrated that IXOSP cleaves chromogenic substrates with arginine in the P_1 position, by a mechanism inhibited by PMSF or aprotinin. Gene expression studies revealed that IXOSP is expressed at different tick developmental stages, including eggs, and unfed or fed adult tick salivary glands, but not in nymphs or in the midgut. While the physiological substrate for IXOSP remains to be identified, we demonstrated that *I. scapularis* saliva activate protein C (PC) resulting in the production of activated PC, a potent anticoagulant that also regulates a myriad of inflammatory responses through protease activated receptors. In contrast, the salivary glands of *Anopheles gambiae, An. stephensi, An. albimanus, Aedes aegypti, Lutzomyia longipalpis, and Phlebotomus ariasi* did not activate protein C. These discoveries are discussed in the context of blood coagulation, inflammation and vector-host interactions.

Keywords

Serine protease; saliva; Ixodes scapularis; protease activated receptors (PAR); activated protein C (APC)

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1. Introduction

Ticks are considered to be second to mosquitoes as vectors of human diseases worldwide (Parola and Raoult, 2001), transmitting a variety of microbes that cause significant human disease with serious public health repercussions to rural, suburban, and even peri-urban populations especially in north-temperate regions of the globe. A better understanding of tick biology may lead to improved strategies for disrupting pathogen transmission and tick feeding on vertebrate hosts (Piesman and Eisen, 2008). Both pathogen transmission and successful blood feeding are dependent on tick saliva which is injected at the site of their attachment to a host to inhibit hemostasis, inflammation, as well as innate and adaptive immune responses (Francischetti et al., 2009). As the tick prepares to insert its hypostome through the skin of the host, contraction of the salivary ducts initiates the flow of saliva to the tip of the mouthparts. Saliva is pumped into the feeding site before any blood is consumed, and thereafter the tick goes through alternating cycles of feeding and salivating which are critical for survival (Stone et al., 1983).

Tick salivary components include amine binding proteins, and cytokine inhibitors which act as anti-hemostatic, anti-inflammatory and immunomodulators, antimicrobials, enzymes and enzyme inhibitors (Francischetti et al., 2009, Kazimirova and Stibraniova, 2013, Mans and Neitz, 2004, Maritz-Olivier et al., 2007, Montgomery et al., 2004, Oliveira et al., 2011, Oliveira et al., 2011, Pichu et al., 2011, Steen et al., 2006). Surprisingly, while insects proteolytic enzymes have been studied in detail (Ashida et al., 1990, Jiang and Kanost, 2000, Lee et al., 2007, Lopes and Terra, 2003), in ticks, there has been very few reports on the function of enzymes in saliva, despite an abundancy of genes coding for serine proteases and metalloproteases in this tissue for several species (Francischetti et al., 2009, Mulenga et al., 2001, Valenzuela et al., 2002). In fact, the only two enzymes biologically identified in *I. scapularis* saliva are metalloproteases, which regulate angiogenesis (Francischetti et al., 2005) and fibrinolysis (Francischetti et al., 2003). Due to the pleiotropic nature of serine proteases in activating anticoagulant, fibrinolysis, or anti-inflammatory processes, these enzymes are likely important for successful blood feeding and digestion and perhaps, pathogen transmission (McNally et al., 2012, Miyoshi et al., 2008, Ribeiro and Francischetti, 2003, Ullmann et al., 2013).

In the course of fractionating *I. scapularis* saliva, we sought to determine amidolytic activity of saliva. A novel proteolytic enzyme was purified as a serine protease (IXOSP) of 29.9 KDa that displays activity compatible with trypsin-like enzymes. We also tested and discovered that tick saliva activates protein C.

2. Materials and Methods

2.1 Source of ticks and blood sucking insects

I. scapularis ticks were collected from forested sites in southern Rhode Island. For some experiments, adult ticks were allowed to feed on New Zealand white rabbits under controlled conditions (Mather and Mather, 1990). A restraining collar was placed around the neck of each rabbit, and their ears were covered with cotton stockinette prior to tick exposure. For these experiments, different development stages of ticks were collected. All animal studies were approved by the University of Rhode Island Institutional Animal Care and Use Committee (protocol number AN01-12-014). *Anopheles gambiae, An. stephensi, An. albimanus, Aedes aegyptii, Lutzomyia longipalpis, and Phebotomus ariasi* were reared at the LMVR/NIAID/NIH.

2.2. Tick saliva collection

Adult-stage ticks weighing 200–300 µg were used for tick saliva extraction. The pilocarpine induction method was used to induce ticks to salivate (Ribeiro et al., 2004). Ticks were permitted to engorge for 4–5 days on the ear of a rabbit, after which they were removed by traction using pointed tweezers. Harvested ticks were rinsed in distilled water and immediately fixed to glass slides with double-sided tape, and a sterile glass micropipette was placed around the hypostome to collect saliva. Salivation was induced by applying 2 µl of pilocarpine (50 µg/ml) in 95% ethanol to the scutum of the tick. Additional 1-µl volumes of pilocarpine were applied at 20-min intervals when little salivation was observed. Ticks were incubated at 35°C in a humid chamber until salivation ceased (2 to 3 h). Micropipettes were removed from the ticks and amount of saliva collected was determined. Typically, volumes of 10 µl per tick were collected. The saliva was pooled and stored at −70°C.

2.3. IXOSP purification

Saliva (300 µl) was diluted with equal volume of Milli Q Water and centrifuged for 10 min at 14,000×g. The supernatant was chromatographed in a HiTrap benzamidine column (GE Healthcare, Piscataway, NJ) using fast-performance liquid chromatography (FPLC) equilibrated in 20 mM Tris-HCl, pH 8.0. The unbound protein was removed by washing buffer containing 0.05 M Tris HCl, 0.5 M NaCl, pH7.4, until absorbance at 215nm was zero. Bound proteins were eluted with 0.05 M glycine, pH 3.0 and the fractions were immediately collected and neutralized in 200 µl of 1 M Tris HCl, pH 9.0. The peak obtained was pooled, concentrated in a speed-vac, and checked for amidolytic activity (see below).

Active fractions were applied into a reverse-phase high-performance liquid chromatography (HPLC) C18 column (0.5 mm × 150 mm) (Phenomenex, Torrance, CA) equilibrated with a flow 5 or 10 µl/min using an ABI 140D pump and 785A UV detector from Applied Biosystems (Foster City, CA). Solution A contained water and 0.1% formic acid (FA), and solution B contained 0.1% FA in acetonitrile. After injecting the sample into the column, a gradient from 10% to 80% B was carried out for 40 min at a flow rate of 10 µl/min. Fractions were collected using the Gilson 203B fraction collector (Gilson, Inc., Middleton, WI) at 1-min volume intervals. Protein values for each of the fractions were measured using a Nano Drop UV/Vis Spectrometer, and amidolytic activity was determined as described below. The amidolytic peak fraction was collected, pooled, and concentrated in a speed-vac. All experiments were carried out with the purified fraction.

2.4. Mass spectrometry (MS)

Molecular mass of the protein was determined by matrix-assisted laser desorption/ionization time-of-flight (MALDI/TOF) MS analysis. The experiments were conducted on a Ciphergen ProteinChip® System (Ciphergen Biosystems, Fremont, CA) using the matrix solution of α-cyano-4-hydroxycinnamic acid (Sigma Chemical, St. Louis, MO) suspended in 100% acetonitrile.

2.5. Amino acid sequencing

N-terminal amino acid sequencing of the isolated protein was obtained by automated Edman degradation. Phenylthiohydantoin derivatives were detected using a pulse liquid automatic sequencer (Applied Biosystems, Fullerton, CA). The resulting sequences were analyzed using the expert protein analysis system (ExPASy) server of the Swiss Institute of Bioinformatics (SIB; Lausanne, Switzerland).

2.6. Sequence analysis

Sequence identity analysis of the serine protease gene was performed using the blastx, and protein by blastp program (Altschul et al., 1997) and comparing it with Genbank/protein bank databases. Analysis for putative signal peptide cleavage sites was carried out with SignalP 3.0 (Bendtsen et al., 2004) and calculations of theoretical isoelectric point was conducted with WINPEP and PROTPARAM (Hennig, 2001). ClustalX 1.83 software (Thompson et al., 1997) was used for multiple sequence alignments, and alignments were displayed with GENEDOC and refined manually.

2.7. Enzyme activity

Enzymatic activity was determined by the release of *p*-nitroanilide (pNA) from the different synthetic substrates (Chromogenix, Milano, Italy). Activity was measured at 405 nm by using an ELISA plate reader (Labsystem Multiskan, USA). The assays were performed in 96-well tissue culture plates in 50 µl of 100 mM Tris-HCl, pH 8.0, and 0.3 mM of different substrates. The samples were incubated at 37°C, and the reaction was stopped by the addition of 25 µl of 30% acetic acid. One enzymatic unit was defined by the release of 1 µmol of pNA per min at 37°C at pH 8.0. In some experiments, protease inhibitors were incubated with the enzymes as described below.

2.8. Characterization of proteolytic activity

All substrates and inhibitors were obtained from Sigma-Aldrich (Taufkirchen, Germany), prepared, and stored according to the manufacturer's instructions. Influences of solvents were excluded by measuring the appropriate solvent only. Optimal pH of proteolytic activity was determined using three independent samples. Briefly, 0.5 µl purified IXOSP was incubated at 37°C for 150 min in a 400 µl reaction mixture (120 mM Britton Robinson buffer pH 4–10, 0.3 mM BAPNA, w/v). Reactions were stopped by adding 400 µl 10% trichloracetic acid (w/v). After 10 min precipitation on ice, samples were centrifuged for 10 min at 13,000×g at 4°C. Supernatant (700 µl) was mixed with 300 µl 5M NaOH, and the optical density was determined at 405 nm. Effects of inhibitors were determined as described above with 0.3 mM BAPNA as substrate. The following substrates were tested: 95 µM PMSF, 100 µM TLCK, 30 µM antipain, 10 µM aprotinin, 1.8 µM SBTI, and 25 µM E-64. Three independent samples were used in duplicate measurements. The effect of reducing agent was determined with 1 mM DTT.

2.9-Proteolytic activity determined by gel electrophoresis or zymograms

Zymogram were performed with SDS-PAGE (12.5%) prepared with 3.0 mg/ml of gelatin (DIFCO; Becton Dickinson and Company, San José, CA) to detect proteolytic activity in the gel (Hanspal et al., 1983). After electrophoresis, gels were washed twice (30 s) with 2% Triton-X-100 to remove SDS and incubated at 37°C in 100 mM Tris-HCl, pH 8.0. After incubation, gels were stained with Coomassie brilliant blue R250 in ethanol:water:acetic acid (4:4:1) and destained with a solution of ethanol:water: acetic acid (4:4:1).

2.10. Tissue expression profile analysis

Gene-specific primers were designed from the tick-specific expressed sequence tag (EST) sequence for IXOSP (GenBank accession number DQ066103) to amplify a cDNA fragment from different stages of tick tissues [20–30 eggs, fed and unfed larvae, fed and unfed nymphs, and fed and unfed adult salivary glands (SGs) and midguts] dissected from all stages of *I. scapularis*. Total RNA was isolated from each tick tissue using an RNAqueous® total RNA isolation kit (Ambion, Inc., Austin, TX). Concentration of total RNA was determined spectrophotometrically at 260 and 280 nm, and aliquots were stored at −80°C until use. Total RNA was reverse transcribed using Maloney murine leukemia virus (M-

MLV) reverse transcriptase according to an Invitrogen kit protocol. For each set, cDNA was PCR amplified using gene-specific primers for IXOSP (Forward: 5′-ATGGGGTTCCATACACGAAA-3′; Reverse: 5′-GTGTAT CCACCCGGCATATC-3′); β-actin (Forward: 5′-AAACATCCGACATGTGTGACGACGA-3′; Reverse: 5′-TGTGGTGCCAGATCTTCTCCATGT-3′) as a control (Stratagene, La Jolla, CA) using the PCR program of 94°C for 4 min, 35 cycles of 94°C for 1 min, 49°C for 1 min, and 72°C for 1 min. All experiments were carried out in triplicate.

2.11. Tick saliva fractionation

Two hundred μl of saliva was loaded in a Sephadex G-75 10/300 column (GE Healthcare Bio-Sciences) equilibrated in PBS, pH 7.4, at 0.5 ml/min. Fractions of 0.5 ml were collected in polypropylene 0.5-ml volume 96-well plates (Costar 3956) and frozen.

2.12. Protein C activation

0.5 μl of *Ixodes* saliva, or 1 pair/assay of the salivary gland homogenate (SGH) from other blood-sucking arthropods, or 20 μl of fractionated saliva was added to 100 μl EBM-2-BSA 0.3% medium (used for endothelial cells culture without FBS or growth factors), supplemented with 100 nM of Protein C (Enzyme Research Laboratory). After overnight incubation, 5 μl of S2236 (250 μM, Chromogenix) was added to the wells, and substrate hydrolysis was detected at 405 nm essentially as described (Collin et al., 2012).

3. Results and Discussion

Several genes coding for serine proteases have been sequenced from *Ixodes scapularis* salivary gland cDNA libraries (Francischetti et al., 2009, Ribeiro et al., 2006, Valenzuela et al., 2002). Following a preliminary assay, saliva was found to hydrolyze small chromogenic substrate for trypsin-like proteases, such as BAPNA. In an attempt to identify the enzyme involved in this activity, saliva was loaded in a benzamidine-affinity column, eluted (not shown), and active fractions were combined and loaded on a reverse-phase HPLC C18 column. Two peaks (1 and 2) were obtained (Fig. 1A). Peak 2 showed maximum amidolytic activity (5.3 Units/mg) and was used for further characterization. Table 1 presents the yield for protein purification steps. Edman degradation identified the N-terminus FPxMVxLRIKxR and MALDI/TOF analysis calculated a molecular weight of 29.9 kDa (Fig. 1B). Purified IXOSP was evaluated by SDS-PAGE revealing that it migrates at a molecular weight of ~29 kDa (Figure 1C).

IXOSP is a serine protease

Based on the N-terminus, a BlastP search showed this protein to be a putative 29.9-kDa secreted protein (AAY66740) from *I. scapularis* salivary gland. Figure 2 shows the open reading frame and translation for IXOSP. The predicted protein exhibits a signal-peptide of 19 aa, followed by 31 amino acid residues which appears to be the result of proteolytic cleavage, and the N-terminus identified by Edman degradation. The mature protein is composed of 223 amino acids (mol mass 24.7 kDa) with serine protease conserved domain motifs, including the catalytic triad **His**, **Asp**, and **Ser**. To confirm that the proteolytic activity of IXOSP exists, zymograms were performed, where gelatinolytic activity was detected at the molecular weight expected for the purified enzyme (Figure 2B). The discrepancy between the predicted mass and the mass calculated by MS is possibly due to glycosylation or other post-translational modifications.

IXOSP exhibits serine protease activity

Next, substrate specificity of IXOSP was tested against several substrates used to detect serine protease activity. As shown in Table 2, the best-cleaved substrate was N-alpha-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as well as N-p-Tosyl-Gly-Pro-Lys-p-nitroanilide, both of which are substrates for trypsin; similarly, IXOSP exhibits specificity for arginine- or lysine-containing substrates at the P1 position. The proteolytic activity of the IXOSP against BAPNA was highest at neutral to slightly basic pH (Table 3).

IXOSP activity was inhibited by DTT, PMSF, aprotinin, soya bean trypsin inhibitor (SBTI), and antipain (Table 4). This inhibitory profile confirms that the enzyme is a serine protease, dependent on disulfide bonds. In contrast, no inhibition was found with cysteine protease inhibitor, E-64. Table 5 compares the amidolytic activity of trypsin and IXOSP.

The function of IXOSP is presently unknown. It is unable to cleave angiotensin and bradykinin, which could mediate pain sensibility. Furthermore, IXOSP has no effect on fibrin clots, i.e., it does not display fibrinolytic activity (data not shown), in contrast to saliva (Francischetti et al., 2003). Given that the maximum activity of IXOSP is at pH 8.0, it also is unlikely that it is involved in a digestive role but more likely targets proteins in the blood, where the pH is about 7.4. It also may cleave other salivary proteins. In fact, some salivary molecules are stored in the glands as inactive zymogens. For example, a trypsin-like protease, triapsin, is stored as a proteolytically activatable zymogen in the lumen *Triatoma* sp. (Amino et al., 2001). In many insects, such activating proteases are often serine proteases, which can be activated either by themselves or by other serine proteases (Kollien et al., 2004).

Gene expression for IXOSP

To estimate the gene expression levels of IXOSP in different tissues of *I. scapularis,* RT-PCR experiments were performed using a specific primer for the enzyme. Figure 3A shows that IXOSP gene expression was observed in eggs in addition to unfed and fed adult tick SGs. IXOSP gene expression was not detectable in whole larvae, or nymphs, or in the midgut of adult ticks, either fed or unfed. Tick β-actin gene was used as a control (Fig. 3B).

Tick saliva activates protein C

While the biological activity of purified IXOSP was not identified in this study, it is known that serine proteases modulate hemostasis through conversion of protein C to activated protein C (APC), a potent anticoagulant (Esmon, 2002). So far, the only molecule that affects APC is a thrombin inhibitor (BmGTI) that enhances the amidolytic activity of the enzyme, but does not activate PC (Ricci et al., 2007). To test whether saliva activates protein C, it was incubated with PC overnight in EBM-2 media, which is used for endothelial cell culture, and therefore contains all divalent ions (e.g. Ca^{2+}, Zn^{2+}) necessary for enzymatic activity. APC activity was detected with S2366 hydrolysis. Figure 4A shows that 1 µl saliva per assay produced substrate hydrolysis, while the SGH from *Ae aegypti*, *Lutzomyia longipalpis*, and *Phlebotomus ariasi* do not. In contrast, SGH from *An. stephensi*, *An. gambiae*, and *An. albimanus* inhibited the basal activity of APC, also suggesting the presence of an inhibitor.

Next, the divalent ion dependency for the activity was studied. Figure 4B demonstrates that 0.5 µl saliva activated PC in Hepes-saline (HS) buffer, but this activity was enhanced by Ca^{2+} and particularly by Zn^{2+}, at levels comparable to results obtained in the presence of EBM-2. EDTA completely abrogated the activity. These results indicate that the molecule involved in PC activation is metal dependent and conceivably this activity is mediated by a serine protease, or metalloproteases. Both enzymes are abundantly expressed in *I. scapularis*

salivary gland based on sialome analyses (Francischetti et al., 2009, Ribeiro et al., 2006, Valenzuela et al., 2002). Activation of PC was dose dependent, since 2 μl saliva more than tripled the activation observed with 0.5 μl. To determine the approximate molecular weight of the PC activator, saliva was loaded in a gel-filtration column, and eluted with PBS. Figure 4C demonstrates that activity for PC activation is found in several fractions containing proteins with molecular weights approximating 30–60 kDa. Activity was not present when saliva was incubated with S2366 in the absence of PC.

Concluding remarks

The findings that a serine protease was purified directly from saliva indicate that these enzymes are expressed in the gland. While the function of this protein was not elucidated, saliva was found to activate protein C. This is a notable finding, and it is the first such report in the salivary gland of any blood sucking arthropod tested so far. Activation of the protein C pathway is a remarkable strategy because APC is a potent anticoagulant through cleavage of FVa and FVIIIa, two cofactors for prothrombinase and intrinsic Xnase complexes, which amplify and consolidate the blood coagulation cascade (Roberts, 2010). In addition, APC down-regulates several pro-inflammatory genes through PAR1 activation in different cell types (Joyce et al., 2001). Discovery that saliva activates PC in addition to several other anticoagulants already identified (Koh and Kini, 2009) indicates that *I. scapularis* exhibits a repertoire of mechanisms which may act in concert to down-modulate the hemostatic process, particularly that of blood coagulation.

Acknowledgments

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A serine protease (IXOSP) was purified from the saliva of the tick Ixodes scapularis

Saliva from tick, but not from mosquitoes, activates Protein C

Salivary enzymes emerge as novel negative modulators of hemostasis
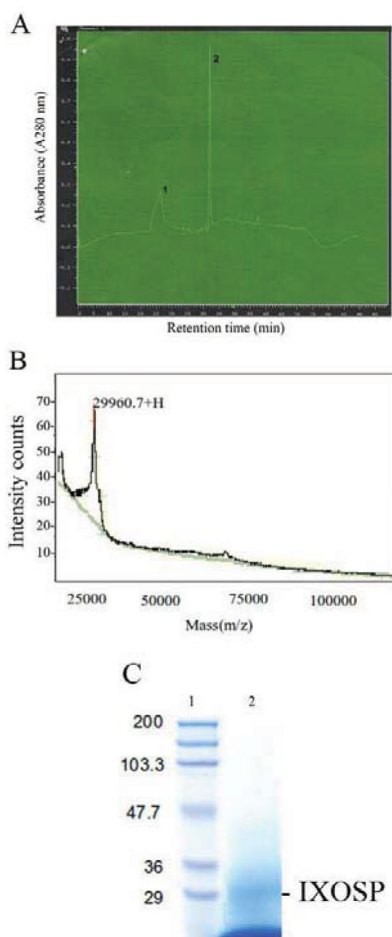
Fig. 1. Purification of IXOSP
(A) Saliva was applied to benzamidine-affinity column chromatography, eluted, and tested for amidolytic activity. Active fractions were applied to a HPLC-Reverse C18 column using a gradient from 10% to 80% acetonitrile, formic acid 0.1% for 40 min at a flow rate of 10 μl/min. The eluate was monitored at 225 nm. All fractions were concentrated in a speed-vac and checked for amidolytic activity. (B) MALDI/TOF MS analysis of purified IXOSP. Spectra were analyzed to the most intense matrix peak at *m/z* 29960.7. (C) SDS-PAGE. Purified IXOSP was loaded in a 12.5% SDS-PAGE. Gels were Coomassie brilliant blue stained. Lanes: 1, Prestained molecular weight marker; 2, HPLC purified protein (peak 2).

(A)

```
  1 atgaagtttgtgacggtagccatcctcgttggaacagtagtcggg
    M  K  F  V  T  V  A  I  L  V  G  T  V  V  G
 46 ctgtcaagtgctaaaggcgttgtccaactggaaagcggtctcgaa
    L  S  S  A  K  G  V  V  Q  L  E  S  G  L  E
 91 tgtggacgaccatcaatcagcgctcgcatcgtgaatggaacgatg
    C  G  R  P  S  I  S  A  R  I  V  N  G  T  M
136 gcctcgattgaaaagtttccctggatggtgaagttacggattaag
    A  S  I  E  K  F  P  W  M  V  K  L  R  I  K
181 tatagagtatggatggcatgctgcggagtcataatcaccgccagg
    Y  R  V  W  M  A  C  C  G  V  I  I  T  A  R
226 cacgtgcttacggctgcacactgcgtaaaacggaacggcagtctg
    H  V  L  T  A  A  H  C  V  K  R  N  G  S  L
271 gagccatcggagatcagggtcagctacggccattccgaacacgaa
    E  P  S  E  I  R  V  S  Y  G  H  S  E  H  E
316 aaaggacaagtactgtctgtgaaggccctctaccgacacaggcat
    K  G  Q  V  L  S  V  K  A  L  Y  R  H  R  H
361 ttcaacgccacaacgtacaatcatgacatcgccatgctcgttcta
    F  N  A  T  T  Y  N  H  D  I  A  M  L  V  L
406 aagacatctctgacactgggcccgacgtcgagacacatctgcctt
    K  T  S  L  T  L  G  P  T  S  R  H  I  C  L
451 ccgagcggaaatcacgccttcggtgatcaaacggcgatcgtggtt
    P  S  G  N  H  A  F  G  D  Q  T  A  I  V  V
496 ggatggggttccatacacgaaagttcaatatatggcgcttcggaa
    G  W  G  S  I  H  E  S  S  I  Y  G  A  S  E
541 ctgaggtacacgagccaggttgtatggccaagcgacaactgcagc
    L  R  Y  T  S  Q  V  V  W  P  S  D  N  C  S
586 gcaaagttgaagttcttcaatccgaaaacgcagatttgcgcttac
    A  K  L  K  F  F  N  P  K  T  Q  I  C  A  Y
631 gaccgctactccgatgcatgtgtgggcgactcgggtggacccctg
    D  R  Y  S  D  A  C  V  G  D  S  G  G  P  L
676 atgataaagaacggcgatgcgttcgaactgatcggcctcgtctcc
    M  I  K  N  G  D  A  F  E  L  I  G  L  V  S
721 agtggcattggctgcaatcgaccagatatgccgggtggatacact
    S  G  I  G  C  N  R  P  D  M  P  G  G  Y  T
766 cggattacgcgctacctgaaatggataaacaaggcactcatccac
    R  I  T  R  Y  L  K  W  I  N  K  A  L  I  H
811 agtaacagctga 822
    S  N  S  *
```

(B)

Fig. 2. cDNA of IXOSP

(A) The full length clone for AY 66740 is depicted. The N-teminus is underlined. The putative activation peptide is boxed, and the N-terminus identified by Edman degradation is italicized. The catalytic triad His (H), Asp (D) and Ser (S) is circled. (B) Zymogram of purified IXOSP. Purified IXOSP was loaded in a 12.5% SDS-PAGE containing 3.0mg/ml of gelatin. Following electrophoresis, the gels were washed with 2% Triton-X-100 to remove SDS and incubated at 37°C in 100 mM Tris-HCl, pH 8.0. Gels were Commassie Blue stained. Lanes: 1, Prestained molecular weight marker; 2, HPLC purified protein (peak 2) from Figure 1A.

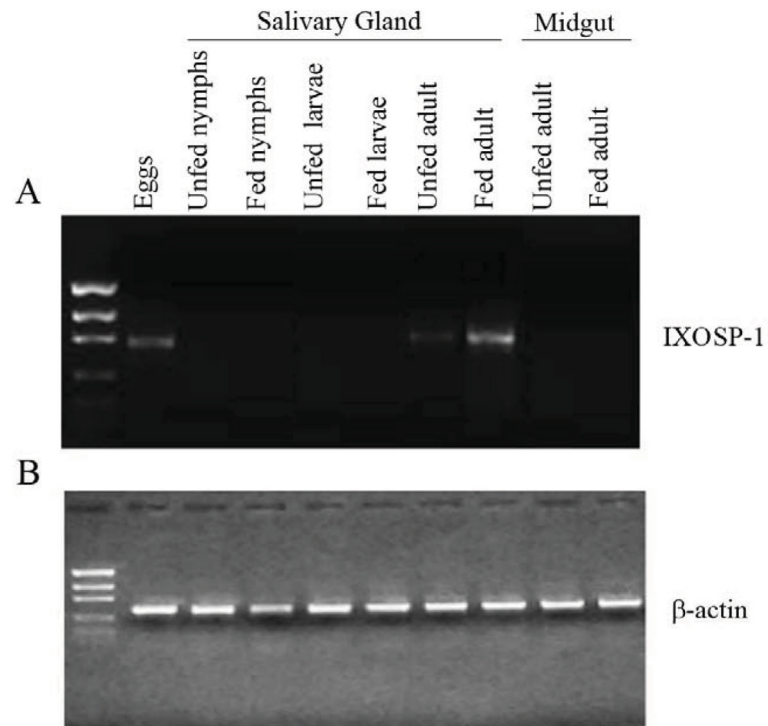**Fig. 3. Gene expression of IXOSP**

(A) RT-PCR was carried out using gene-specific primers for IXOSP. Total RNA was extracted from tissues from different developmental stages of ticks. All experiments were carried out in triplicate. cDNA from different tick tissues were PCR amplified using gene-specific primers fro (A) IXOSP or (B) β-actin. The tissues used for RNA extraction are indicated in the figure.
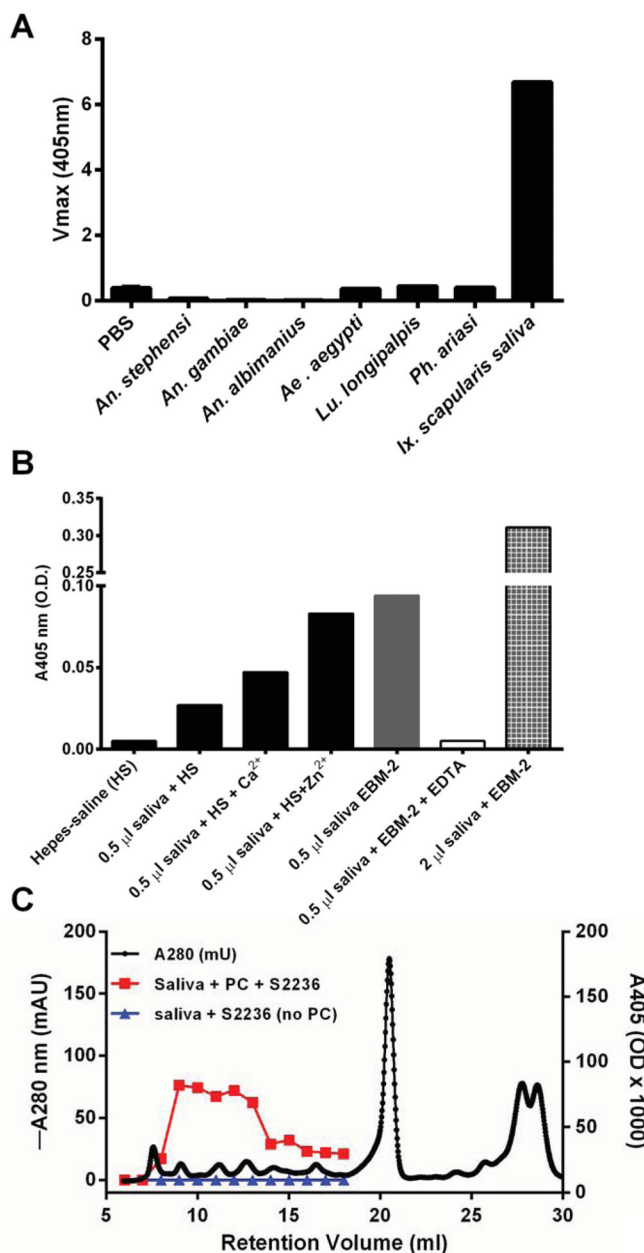
Fig. 4. *I. scapularis* saliva activates protein C
(A) Saliva (1 μl) of *I. scapularis* or SGH (1 pair/assay) from different mosquitoes were incubated with PC (100 nM) overnight in EBM-2 media (100 μl, total volume), followed by addition of substrate specific for PC, S2366 (250 μM). Substrate hydrolysis was detected at 405 nm. (B) Saliva (0.5 μl) was incubated with PC in Hepes saline, 0.3 % BSA overnight, in the presence of $CaCl_2$ (10 mM), $ZnCl_2$ (100 μM) and compared to activation carried out in the presence of EBM-2 containing 0.3% BSA. In some experiments EDTA was added to chelate divalent ions. (C) Saliva (200 μl) was loaded in a G-75 chromatography column, and eluted with PBS. Twenty μl of each fraction was incubate with 100 nM PC overnight in EBM-2-BSA, and S2366 hydrolysis was estimated at 405 nm (squares). As a control, 20 μl of saliva was incubated with S2366, in the absence of PC (up triangle).

Table 1

Purification profile of IXOSP.[a]

Sl. No.	Sample	Total protein (mg/ml)	Activity (BAPNA) [b]	Specific activity of enzyme (Units/mg)
1	Saliva	0.97 ± 0.08	0.48 ± 0.07	0.494
2	Protein peak I (HPLC)	0.01 ± 0.001	0.02 ± 0.001	2.0
3	Protein peak II (HPLC)	0.03 ± 0.002	0.16 ± 0.01	5.3

[a] Protease activity was assayed by using BAPNA as a substrate. Values are mean ± SD for three experiments. The peak fractions were assayed as described in Materials and Methods. The assay was monitored at 405 nm.

[b] 1 unit, 1 μl of pNA released per min at 37°C.

Table 2

Hydrolysis of chromogenic substrates by IXOSP.[a]

Substrate	Substrate specific for	Specific activity(Units/mg) [b] (Purified protein IXOSP)
Benzoyl-DL-Arg-p-nitroanilide (BAPNA)	Trypsin	5.32 ± 0.40
N-p-Tosyl-Gly-Pro-Lys-p-nitroanilide	Trypsin	5.12 ± 0.50
H-D-Val-Leu-Lys-p-nitroanilide	Plasmin	0.140 ± 0.02
Suc-Ala-Ala-Pro-Phe-Arg-p-nitroanilide	Chymotrypsin	2.18 ± 0.20
N-p-Tosyl–Gly-Phe-Arg-p-nitroanilide	Thrombin	1.83 ± 0.11

[a]IXOSP purified by the HPLC column was incubated with the synthetic substrates listed above in a concentration of 0.3 mM in Tris-HCl, 100 mM, pH 8.0, at 37°C. The numbers are mean ± standard deviation of triplicate measurements.

[b](Units/mg = μmol of pNA released per min per mg of protein).

Table 3

Hydrolysis of BAPNA by IXOSP at different pH values.

pH	Average±SD Specific activity(Units/mg)[a]	IXOSP % activity
4	0.523 ± 0.02	10
5	0.784 ± 0.06	15
6	2.615 ± 0.1	50
7	4.707 ± 0.3	90
8	5.235 ± 0.5	100
9	3.664 ± 0.2	70
10	1.046 ± 0.1	20

0.5 µl purified IXOSP was incubated at 37°C for 150 min in a 400 µl reaction mixture. Reactions were performed as discussed in Materials and Methods. The numbers are mean ± standard deviation of triplicate measurements.

[a](Units/mg = µmol of pNA released per min per mg of protein).

Table 4

Effects of protease inhibitors on proteolytic activity of IXOSP.[a]

Sl.No	Inhibitor [b]	Concentration (μM)	Inhibition of activity (% ± SD)
1	Antipain	30	55 ± 4 *
2	SBTI	1.8	72 ± 10 *
3	E-64	25	n.i.
4	Aprotinin	10	77 ± 10 *
5	APMSF	95	75 ± 7 *
6	DTT	100	85 ± 2

[a]Effects of different inhibitors on the proteolytic activity of IXOSP. Experiments were carried out according to Materials and Methods Mean values significantly different from the proteolytic activity in the IXOSP are indicated by * ($P < 0.001$).

[b]TLCK, tosyl-L-lysyl chloromethane hydrochloride; SBTI, soya bean trypsin inhibitor; APMSF, 4-amidinobenzyl methane sulphonyl fluoride hydrochloride, DTT, Dithiothreitol.

[c]n.i., not inhibited.

Table 5

Comparison of hydrolysis profile.[a]

Substrate	Trypsin	IXOSP
Ac-Ile-Glu-Gly-Arg-pNA	1.00	0.82
H-D-Ile-Pro-Arg-pNA	0.24	1.00
H-D-val-Leu-Arg-pNA	0.12	0.03
H-D-Val-Leu-Lys-pNA	0.01	<0.01

[a]Data for trypsin were extracted from Chromogenix catalog and normalized as 1 toward the best substrate.