Identification and characterization of the plasma kallikrein-kinin system inhibitor, haemaphysalin, from hard tick, *Haemaphysalis longicornis*

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Summary

The plasma kallikrein-kinin system inhibitor, haemaphysalin, from the hard tick, *Haemaphysalis longicornis*, was identified. It was found that haemaphysalin inhibited activation of the plasma kallikrein-kinin system by interfering with reciprocal activation between factor XII and prekallikrein. It did not, however, inhibit amidolytic activities of factor XIIa and kallikrein. Direct binding assay indicated that factor XII/XIIa and high molecular weight kininogen (HK) are the target molecules of haemaphysalin, and that Zn²⁺ ions are involved in the interactions of haemaphysalin with these target molecules. This suggests that haemaphysalin interacts with target molecules by recognizing their conformational changes induced by Zn²⁺ ions. Furthermore, haemaphysalin interacted with the fibronectin type II domain and domain D5, the cell binding domains of factor XII and HK, respectively. This finding suggests that haemaphysalin interferes with the association of factor XII and the prekallikrein-HK complex with a biologic activating surface by binding to these cell-binding domains, leading to inhibition of the reciprocal activation between factor XII and prekallikrein.

Keywords

Kunitz-type protease inhibitor, plasma kallikrein-kinin system, tick, salivary gland

Introduction

The kallikrein-kinin system of human plasma consists principally of three plasma proteins, factor XII, prekallikrein, and high molecular weight kininogen (HK). Tissue injury activates this system *in vivo*. Activation of the plasma kallikrein-kinin system is followed by reciprocal activation between factor XII and prekallikrein, leading to the generation of factor XIIa and kallikrein in (1). The generated kallikrein cleaves HK, releasing bradykinin, a primary mediator of inflammation reactions. Bradykinin induces vasodilation, increases microvessel permeability, and enhances sensitivity to pain, resulting in inflammatory symptoms such as redness, edema, and pain around the injured site (2). The generated factor XIIa, on the other hand, catalyzes activation of factor XI, initiating the intrinsic blood coagulation pathway. The physiologic significance of this pathway in the initiation of blood coagulation *in vivo*, however, is questionable, because deficiencies of factor XII, prekallikrein, and HK are not associated with hemorrhagic diathesis (3). An important role of the plasma kallikrein-kinin system, therefore, might be the initiation of mammalian acute-inflammatory responses.

*In vivo*, the binding of the prekallikrein-HK complex to a biologic activating surface, such as endothelial cells, initiates activation of the plasma kallikrein-kinin system (4). On the cell surface, prekallikrein is converted to kallikrein by prolylcarboxypeptidase (5–7) and the generated kallikrein converts factor XII to factor XIIa. *In vitro*, the binding of factor XII to a negatively-charged surface initiates activation of this system. On a negatively-charged surface, factor XII is autoactivated to factor XIa, which catalyzes prekallikrein activation.

The binding of factor XII and HK to biologic activating surfaces requires the divalent cation zinc (8–11). Zn²⁺ ions induce conformational changes in factor XII and HK (12–14), which are thought to be essential for the binding of factor XII and HK to a biologic activating surface, such as the endothelial cell surface. Factor XII and HK compete for the biologic activating surface in
the presence of Zn$^{2+}$ ions, suggesting that factor XII and HK interact with the putative common receptor on the surface via their cell-binding domains (15–17).

Tick saliva contains various pharmacologically active compounds, such as anticoagulants (18), immunosuppressive substances (19), complement inhibitors (20), and platelet aggregation inhibitors (21, 22). When ticks feed on blood, these substances are injected under the host’s skin and counteract host defense responses that impede blood feeding (23). Some Kunitz-type proteins have been identified from ticks. A factor VIIa-tissue factor inhibitor, ixolaris (24), and a factor Xa inhibitor, TAP (tick anticoagulant peptide) (25), were identified from *Ixodes scapularis* and *Ornithodoros moubata*, respectively. The platelet $\alpha_\text{IIb}\beta_3$-integrin antagonist, savignygrin, was found in *O. savignyi* (26). These Kunitz-type proteins might be injected from tick salivary glands into the host skin during blood feeding and act as antihaemostatic molecules. Recently, a large number of Kunitz-type proteins was identified in the cDNA from *I. scapularis* salivary glands by mass sequence analysis, suggesting that tick saliva contains a wide variety of Kunitz-type proteins (27).

In the present study, we performed a mass sequence analysis of salivary gland cDNA from *Haemaphysalis longicornis*, and identified a Kunitz-type protein, designated haemaphysalin. Haemaphysalin inhibits activation of the plasma kallikrein-kinin system by interfering with the reciprocal activation between factor XII and prekallikrein. Haemaphysalin exerts this activity not by affecting the amidolytic activities of factor XIIa and kallikrein, but by interfering with factor XII and HK binding to biologic activation inhibitors (21, 22). When ticks feed on blood, these sub- 

**Materials and methods**

**Materials**

Factor IXa, factor Xa, factor XIa, factor XII, factor XIla, prekallikrein, kallikrein, thrombin, and HK were purchased from Enzyme Research Laboratory (South Bend, IN). To determine protein concentrations, the following extinction coefficients (E 1% 280) and molecular weights were used: factor IXa, 14.9, 56000; factor Xa, 11.6, 46000; factor XIa, 13.1, 160000; factor XII, 14.1, 80000; factor XIla, 14.1, 80000; prekallikrein, 11.7, 86000; kallikrein, 11.7, 86000; thrombin, 18.3, 37000; and HK, 7.01, 120000. Citrated human normal plasma was purchased from Bio Mérieux S.A. (Marcy l’Etoile, France). Corn trypsin inhibitor (CTI) was obtained from Calbiochem (San Diego, CA). Dextran sulfate of MW 50000 (DS500) and soybean trypsin inhibitor (SBTI) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Chromogenic substrates (S-2222, Bz-Ile-Glu-Gly-Arg-p-nitroanilide; S-2238, H-D-Phe-Pip-Arg-p-nitroanilide; S-2302, H-D-Pro-Phe-Arg-p-nitroanilide; S-2366, and pyro-Glu-Pro-Arg-p-nitroanilide) were purchased from Chromogenix AB (Mõndel, Sweden). Spectrozyme® fXa (MeSO$_4$-D-CHG-Gly-Arg-p-nitroanilide) was obtained from American Diagnostica Inc. (Greenwich, CT). Other reagents used in this study were obtained from Wako Pure Chemical Industries, Ltd. and Nacalai Tesque.

**Salivary gland cDNA library construction**

Poly A (+) RNA was isolated from salivary glands of ticks at three different feeding stages; unfed, slow, and rapid feeding stages. Briefly, the MicroPrep mRNA purification kit (Amer- sham Bioscience, Uppsala, Sweden) was used to isolate mRNA. Three cDNA libraries were constructed from the mRNA from each stage using the Superscript plasmid system (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Randomly picked cDNA clones from these libraries were sequenced using ABI PRISM BigDye Terminator cycle sequencing kits and the ABI 310 genetic analyzer (PE Biosystems, Foster City, CA). Sequence similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) program (http://www.blast.genome.ad.jp). Secretory signal peptide and its cleavage-site were predicted using the SignalP program (http://www.cbs.dk/services/SignalP).

**Expression and purification of recombinant protein**

A DNA fragment encoding the predicted mature region of haemaphysalin was amplified using a set of specific primers (forward primer: 5’-CATATGAAATATCGAGCCAACCCAGCACTC-3’; reverse primer: 5’-GGATCCCTAGCGCGGTAAGTACCTTGGACG-3’) and cloned into the *Nde* I – *BamH* I site of the expression vector, pET22-b (Novagen, Darmstadt, Germany). After verifying the DNA sequence of the constructed plasmid, it was introduced into the *E. coli* BL21 (DE3) strain, and production of the recombinant protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside. *E. coli* cells expressing recombinant protein were harvested and suspended in 50 mM Tris-HCl, pH 8.0, containing 15 μM peptatin A, 15 μM leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride, and frozen at −20 °C, then thawed in ice-cold water and sonicated. The cell lysate was centrifuged at 12000 x g for 20 min at 4°C to obtain the inclusion body. The obtained inclusion body was washed with 1 M sucrose, then with 2% Triton X-100.

The recombinant protein from the inclusion body was refolded as previously described by Altman et al (28). Briefly, the inclusion body was solubilized in 25 mM Tris-HCl, pH 8.0, containing 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 7 M guanidine hydrochloride, then diluted 10 times with 100 mM Tris-HCl, pH 8.7, containing 200 mM KCl, 1 mM EDTA, 10 mM reduced glutathione, and 1 mM oxidized glutathione. The refolding reaction was performed by stirring the diluted mixture overnight at room temperature. The refolded protein was dialyzed against 50 mM Tris-HCl, pH 7.5, and purified by gel filtration chromatography using a Sephadex G-75 column (φ 1.8 x 90 cm) equilibrated with the same buffer. The purity of the recombinant protein was evaluated by reverse phase-HPLC (RP-HPLC) analysis. The free SH-group in the purified recombinant protein was titrated using N-(7-dimethylamino-4-methyl-3-coumarinyl)-maleimide (DACM) (29). Purified recombinant protein (100 pmol) was incubated in 200 mM Tris-HCl, pH 8.5, 20 mM EDTA, 6 M guanidine hydrochloride, and 50 mM DACM for 1 h at 20°C. After incubation, the mixture was diluted 5-fold by the addition of H$_2$O$_2$, and then fluorescence intensity at 470 nm...
was measured at an excitation wavelength of 400 nm. The SH-group in the recombinant protein was quantitated using a calibration curve with 2-mercaptoethanol as the standard.

The recombinant fibronectin type II domain of factor XII was expressed in the E. coli codon-plus RIL strain using pET22-b. The expressed fibronectin type II domain was purified by ion exchange chromatography using a Mono S HR5/5 column (Amersham Bioscience) followed by gel filtration chromatography on a TSK2000SW column (TOSOH, Japan). The recombinant domain D5 of HK was prepared according to Herwald et al (14). The domain D5 was expressed in the E. coli BL21 (DE3) strain using pET22-b, and purified by affinity chromatography on a Ni-nitrilotriacetic acid sepharose column followed by gel filtration chromatography on a TSK2000SW column.

Three peptides, HK383–420, HK421–466, and HK459–513, were named after the amino acid sequence of HK, and produced as a glutathione S-transferase (GST) - fused recombinant protein using pGEX6P-1 and the E. coli BL21 strain. All GST-fused recombinant proteins were purified using a glutathione sepharose 4B column (Amersham Bioscience), then digested with PreScission™ protease to remove GST according to the manufacturer’s instructions (Amersham Bioscience). After digestion, recombinant HK383–420, HK421–466, and HK459–513 were further purified using a Wakosil 5C4 column (Wako Pure Chemical Industries, Ltd.).

**Assay for the effects of haemaphysalin on plasma coagulation**

Citrated human normal plasma (20 μl) and recombinant haemaphysalin (30 μl) were preincubated for 5 min at 37°C. Mixtures were activated for 2 min with 30 μl of 25% actin (Dade Behring, Liederbach, Germany) in the activated partial thromboplastin time (APTT) assay and with 30 μl of rabbit brain thromboplastin (Ortho-Clinical Diagnostics, Inc., Raritan, NJ) in the prothrombin time (PT) assay. Clotting reactions were started by adding 25 μl of 50 mM CaCl₂ and the clotting time was measured using a KC-10 coagulometer (Heinrich Amelung, Germany) (30).

**Assay for haemaphysalin inhibitory activity against coagulation factors**

Haemaphysalin inhibitory activity against coagulation factors, factor XIIa, factor XIa, factor Xa, factor IXa, kallikrein, and thrombin, was assessed using several chromogenic substrates. Assays for inhibition against factor XIIa and kallikrein were performed according to Ulmer et al (31). Factor XIIa (final concentration 1.6 nM) or kallikrein (1 nM) was mixed with haemaphysalin at a 1:1000 molar ratio (protease/haemaphysalin) in 50 mM Tris-HCl, pH 7.4, 20 mM NaCl, 2 mM CaCl₂, and 0.005% Triton X-100, and incubated for 1 h at 37°C. After incubation, 20 μl of S-2302 (final concentration 0.5 mM) was added to the mixture. The mixture was incubated for 15 min and the change of absorbance at 405 nm was monitored. Inhibition against factor XIIa, factor Xa, and thrombin was assessed as follows. Factor XIIa (1 nM), factor Xa (1 nM), or thrombin (1 nM) were mixed with haemaphysalin at a 1:1000 molar ratio (protease/haemaphysalin) in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and incubated for 15 min at 37°C. Suitable chromogenic substrates for each assay were used as follows: S-2366 for the factor XIIa assay; S-2238 for the factor Xa assay; S-2238 for the thrombin assay. These substrates (20 μl; final concentration 340 μM) were added to each mixture and incubated for 30 min. After incubation, absorbance changes at 405 nm were measured. The inhibition assay for factor IXa was performed according to the manufacturer’s instructions with some modification using Spectrozyme® FIIXa as a substrate. Factor IXa (80 nM) was mixed with haemaphysalin at a 1:1000 molar ratio (factor IXa/haemaphysalin) in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM CaCl₂, with 33% (v/v) ethylene glycol. The mixture was incubated for 5 min at 37°C, 20 μl of 7.5 mM Spectrozyme® FIIXa was added, and after 20 min of incubation, the absorbance change at 405 nm was monitored.

**Assay for the effects of haemaphysalin on activation of surface-activated plasma zymogen**

The inhibitory activity of haemaphysalin against the generation of factor Xla, factor XIIa, and kallikrein, was measured using human plasma (32–35). Briefly, citrated human normal plasma was treated with acid or acetone to inactivate plasma serine protease inhibitors, then diluted with 1:30 in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 0.2% polyethylene glycol 8000 (PEG8000). The acid-treated plasma was used for the factor XIIa generation assay and the acetone-treated plasma was used in factor XIa and kallikrein generation assays. The diluted plasma (50 μl) and various concentrations of haemaphysalin (20 μl) were mixed and incubated for 5 min at 37°C. After incubation, the surface-activated plasma zymogens, factor XII, prekallikrein, and factor XI, were activated by the addition of 20 μl of 20% actin. After 5 min, the chromogenic substrate (final concentration 340 μM) and serine protease inhibitors (20 nM) were added. The mixture was incubated for 30 min and amidolytic activity of the generated protease (factor XIa, factor XIIa, and kallikrein) was photometrically measured at 405 nm. The sets of chromogenic substrate (340 μM) and serine protease inhibitors (20 nM) in each assay were as follows: S-2366, SBTI, and CTI for factor XIa assay; S-2302 and SBTI for factor XIIa assays; S-2302 and CTI for the kallikrein assay.

**Assay for the effect of haemaphysalin in a reconstitution assay**

The reconstitution assays of the kallikrein-kinin system were performed using purified coagulation factors, factor XIIa/IIIa and prekallikrein/kallikrein (36, 37). To assay for the effect of haemaphysalin on the activation of factor XII by kallikrein, factor XII (final concentration 20 nM) was preincubated with haemaphysalin in buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1% BSA, and 0.1% PEG8000) for 10 min at room temperature. The activation was initiated by the addition of kallikrein (0.2 mM) and DS500 (0.3 μg/ml). After incubation for 10 min, SBTI (0.6 μM) and S-2302 (340 μM) were added, and the increase in absorbance at 405 nm was monitored at 5-min intervals. To assay for the effect of haemaphysalin on prekallikrein activation by factor XIIa, factor XIIa (50 μM) was preincubated with haemaphysalin in the same buffer for 10 min at room temperature. Prekallikrein (10 nM) and DS500 (0.1 μg/ml) were added to the mixture, and then prekallikrein activation started. After a 5-min incubation, CTI (100 nM) and S-2302 (170 μM) were added, and the increase of absorbance at 405 nm was recorded, as described.
above. To assay for the effect of haemaphysalin on the reciprocal activation between factor XIIa and prekallikrein, factor XII (0.2 nM) was preincubated with haemaphysalin in the same buffer for 10 min at room temperature. The autoactivation of factor XII and following reciprocal activation were started by the addition of prekallikrein (10 nM) and DS500 (0.2 μg/ml). After a 10-min incubation, S-2302 (170 μM) was added and an increase in absorbance at 405 nm was recorded at 5-min intervals.

**Binding analysis with surface plasmon resonance**

Interactions between haemaphysalin and coagulation factors were monitored using a BIAcore 3000 instrument (BIAcore AB, Sweden). Haemaphysalin (30 μM) was immobilized onto a sensor chip CM4 in 10 mM acetate buffer, pH 5.0, using an amine coupling kit according to the manufacturer’s instruction. Approximately 2000 resonance units (RU) of immobilized haemaphysalin was used in all assays. The exact amounts of immobilized haemaphysalin in each assay are indicated in the figures. The blank flow cell for the control experiment was prepared using the same procedure, but with no haemaphysalin. Screening of interactions between haemaphysalin and coagulation factors was performed at 25°C in HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM CaCl₂, and 0.005 % Tween 20). Each coagulation factor (100 nM) was injected at 20 μl/min and association was monitored for 2 min. After termination injection of the coagulation factor, dissociation was monitored for 2 min. Generation of the sensor chip after each assay was achieved by a pulse injection of 50 mM EDTA and 1 M NaCl. To investigate the interaction of haemaphysalin with proteins involved in the kallikrein-kinin system, HBS buffer containing 50 μM or 10 μM ZnCl₂ was used for the assays. The interactions with domains and peptides derived from factor XIIa and HK were also examined in the same buffer. HBS buffer was treated using Chelex 100 (Biorad, Hercules, CA) to avoid contamination by metal ions prior to the addition of ZnCl₂. Furthermore, to remove metal ions from samples, the samples were dialyzed before use against a metal-chelated HBS buffer containing 0.1% Chelex 100. The assay procedure was the same as described above. Kinetic constants were evaluated from obtained sensorgrams using BIAevaluation 3.0 software installed in the BIAcore 3000.

**Results**

**Cloning, expression, and purification of haemaphysalin**

A cDNA library was constructed from the salivary glands of ticks at the rapid feeding stage. Approximately 800 cDNA clones were randomly picked up from this library and sequenced. Sequence similarity searches identified a cDNA clone with similarity to a Kunitz-type protease inhibitor, named “haemaphysalin” (Fig. 1). This clone had a 489 bp open reading frame encoding a 162 amino acid protein with a predicted 22 amino acid N-terminal signal peptide. The mature protein contained 140 amino acids, including 10 cysteines, and had a calculated molecular mass of 16222 kDa. Haemaphysalin has two Kunitz-type protease inhibitor domains; the first has six cysteine residues conserved among the Kunitz-type protease inhibitors, and the second has only four.

To investigate the biologic activity of haemaphysalin, the recombinant protein was produced in *E. coli*. The recombinant protein was harvested as an inclusion body and refolded according to a thiol-disulfide shuffling system, developed by Altman et al for refolding of the bovine pancreatic trypsin inhibitor (BPTI), a well known Kunitz-type protease inhibitor (28). After refolding, the recombinant protein was purified by gel filtration chromatography, and purity was confirmed using RP-HPLC.

To investigate the disulfide bond formation of the recombinant protein after refolding, the SH-group derived from the free Cys residue was titrated using the fluorescence reagent, DACM. There were no free SH-groups in the purified recombinant protein (data not shown). In addition, the electrophoretic migration of the protein did not differ under reducing or non-reducing conditions in sodium dodecyl sulfate-polycrylamide gel electrophoresis analyses (data not shown). These results suggested that all the Cys residues in the refolded recombinant protein formed disulfide bonds intramolecularly.

**Haemaphysalin inhibits activation of the plasma kallikrein-kinin system**

Some Kunitz-type protease inhibitors obtained from the salivary glands of ticks were identified as anticoagulants (24, 25, 38). Thus, we first examined the effect of haemaphysalin on APTT and PT using normal human plasma. Haemaphysalin dose-dependently prolonged both APTT and PT, indicating that it could...
inhibit the plasma clotting reaction (Fig. 2A and B). These assays further demonstrated that haemaphysalin prolonged APTT at nanomolar concentration and PT at micromolar concentration, indicating that haemaphysalin affected APTT more than PT.

To investigate the mode of action of haemaphysalin, we examined the haemaphysalin inhibition against several coagulation factors (kallikrein, factor XIIa, factor Xa, and thrombin) using appropriate synthetic chromogenic substrates. Haemaphysalin did not inhibit amidolytic activities of any these coagulation factors, even with the addition of excess haemaphysalin (data not shown). We further examined the ability of haemaphysalin to bind to coagulation factors using surface plasmon resonance (SPR) spectroscopy. This assay clearly demonstrated that haemaphysalin specifically bound to factor XIIa (Fig. 3). Interactions between haemaphysalin and other activated coagulation factors were not evident (Fig. 3).
Because factor XIIa is involved in the activation of the plasma kallikrein-kinin system, haemaphysalin might inhibit activation of the plasma kallikrein-kinin system by interfering with the function of factor XIIa. We examined the effect of haemaphysalin on activation of the kallikrein-kinin system in human plasma. Haemaphysalin dose-dependently inhibited the generation of factor XIa, factor XIIa, and kallikrein (Fig. 4). This indicated that haemaphysalin inhibits activation of the kallikrein-kinin system and thus prolongs APTT.

We further investigated the effect of haemaphysalin on the kallikrein-kinin system in reconstitution assays. Haemaphysalin inhibited the reciprocal activation between factor XII and prekallikrein in a dose-dependent manner (Fig. 5A). In addition, haemaphysalin dose-dependently inhibited the activation of both factor XII and prekallikrein catalyzed by kallikrein and factor XIIa, respectively (Fig. 5B and C). These results indicate that haemaphysalin inhibited all steps of reciprocal activation between factor XII and prekallikrein.

Haemaphysalin interacts with factor XII/XIIa and high molecular weight kininogen

Zn\(^{2+}\) ions are involved in the activation of the kallikrein-kinin system. We investigated the interaction between haemaphysalin and proteins involved in the kallikrein-kinin system in the absence and presence of Zn\(^{2+}\) ions. In the absence of Zn\(^{2+}\) ions, haemaphysalin bound only to factor XIIa (Fig. 6A). In the presence of Zn\(^{2+}\) ions, haemaphysalin also bound to factor XII and HK (Fig. 6B-D). The binding of haemaphysalin to factor XIIa was stronger than to factor XII and HK. Dissociation of the haemaphysalin-
physalin-factor XIIa complex was greatly affected by Zn$^{2+}$ ions (Fig. 6A and B), and the binding became tighter with the addition of Zn$^{2+}$ ions.

In the absence of Zn$^{2+}$ ions, the kinetic constants ($K_{D1}$, $k_a$, $k_d$) of the interaction between haemaphysalin and factor XIIa fit well to a simple 1:1 Langmuir binding model (Table 1).

$$k_a \quad A+B \leftrightarrow AB \quad k_d$$

In the presence of Zn$^{2+}$ ions, however, a simple 1:1 Langmuir binding model did not fit well for analyses of those interactions. A two-state binding model was suitable for the kinetic analyses of the interactions (Table 1).

$$k_{a1} \quad k_{a2}$$

$$A+B \leftrightarrow AB \leftrightarrow (AB)^*$$

$$k_{d1} \quad k_{d2}$$

The obtained kinetic constants led us to assume that haemaphysalin rapidly formed initial protein-protein complexes with factor XII, factor XIIa, or HK in the presence of Zn$^{2+}$ ions, and then gradually becomes a tightly-bound, slowly-dissociating final complex.

Consequently, these SPR analyses demonstrated that the target molecule of haemaphysalin was not only factor XIIa, but also factor XII and HK, and that Zn$^{2+}$ ions were involved in the interaction between haemaphysalin and these target molecules.

**Haemaphysalin binds the fibronectin type II domain of factor XII and domain D5 of high molecular weight kininogen**

Zn$^{2+}$ ion-binding regions of factor XII and HK are located in the fibronectin type II domain and domain D5, respectively (39, 40). Thus, we determined if haemaphysalin could bind to these domains of factor XII and HK. Haemaphysalin interacted with both the fibronectin type II domain and domain D5 in the presence of Zn$^{2+}$ ions (Fig. 7). In contrast, haemaphysalin barely interacted with these domains in the absence of Zn$^{2+}$ ions. In this assay, the interaction of haemaphysalin with domain D5 was stronger than that with the fibronectin type II domain. This, however, might be due to instability of the recombinant fibronectin type II domain. A two-state binding kinetic model fit well for the analysis of these interactions (Table 2). These results indicate that the binding sites of haemaphysalin on factor XII and HK molecules were in the fibronectin type II domain and domain D5, respectively.

**Haemaphysalin interacts with Zn$^{2+}$-ion binding region and cell-binding region on domain D5**

Domain D5 of HK contains a Zn$^{2+}$-ion binding region and a cell-binding region. His$^{441}$-His$^{457}$ and His$^{479}$-His$^{498}$ on domain D5 are

<table>
<thead>
<tr>
<th>-Zn$^{2+}$ ion</th>
<th>+Zn$^{2+}$ ion</th>
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<tr>
<td>$k_a$</td>
<td>$k_d$</td>
</tr>
<tr>
<td>M$^{-1}$S$^{-1}$ (x10$^3$)</td>
<td>S$^{-1}$ (x10$^3$)</td>
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<tr>
<td>Factor XII</td>
<td>N.D.</td>
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<tr>
<td>Factor XIIa</td>
<td>2.23 ± 0.66</td>
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<tr>
<td>HK</td>
<td>N.D.</td>
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N.D.: Not determined because haemaphysalin did not interact with factor XIIa and HK in the absence of Zn$^{2+}$ ions.

Figure 7: Sensorgrams for interactions of haemaphysalin with fibronectin type II domain and domain D5. Haemaphysalin was coupled onto the sensor chip at levels of 2543 RU and 1781 RU in the binding assays for fibronectin type II domain (A) and domain D5 (B), respectively. Assays were performed at a flow rate of 20 μl/min in the presence of 50 μM ZnCl$_2$. Regeneration was achieved using 50 mM EDTA and 1 M NaCl. The insets indicate the sensorgrams obtained from the interaction of haemaphysalin with fibronectin type II domain and domain D5 in different concentrations of Zn$^{2+}$ ions (0, 10, 50 μM).
Table 2: Kinetic constants of interactions of haemaphysalin with fibronectin type II domain and domain D5. Kinetic constants were evaluated from sensorgram curves using kinetic evaluation software installed in the BIAcore 3000 system. The kinetic fitting models used in the assay were two-state binding model.

<table>
<thead>
<tr>
<th></th>
<th>( k_{a1} ) (M(^{-1})S(^{-1}))</th>
<th>( k_{a2} ) (S(^{-1}))</th>
<th>( k_{d1} ) (M(^{-1})S(^{-1}))</th>
<th>( k_{d2} ) (S(^{-1}))</th>
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<tr>
<td>Fibronectin type II domain</td>
<td>6.41 ± 0.97x10(^{-4})</td>
<td>1.59 ± 0.55x10(^{-3})</td>
<td>2.11 ± 1.37x10(^{-4})</td>
<td>1.06 ± 0.06x10(^{-4})</td>
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<tr>
<td>Domain D5</td>
<td>1.61 ± 0.02x10(^{-3})</td>
<td>7.30 ± 2.21x10(^{-2})</td>
<td>4.21 ± 1.71x10(^{-2})</td>
<td>5.20 ± 0.38x10(^{-2})</td>
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Discussion

From *Haemaphysalis longicornis*, we identified haemaphysalin, a plasma kallikrein-kinin inhibitor that shares sequence similarity with Kunitz-type protease inhibitors. Haemaphysalin inhibited all steps in the reciprocal activation between factor XII and prekallikrein without affecting the amidolytic activities of proteases involved in this reaction (kallikrein and factor XIa). Because typical Kunitz-type protease inhibitors act by inhibiting the amidolytic activity of target coagulation factors (25, 38), these results indicate that haemaphysalin uses unique mechanisms to inhibit the activation of the plasma kallikrein-kinin system.

SPR analysis revealed that haemaphysalin interacts with factor XII/XIa and HK, especially with cell-binding domains of both molecules, domain D5 of HK and fibronectin type II domain of factor XII/XIa. Haemaphysalin binds directly to the cell-binding region (HK459–513) on domain D5. This result strongly suggests that the binding interferes with association of the prekallikrein-HK complex with the activating surface. Based on these results, haemaphysalin likely prevents both the factor XII and the prekallikrein-HK complex from associating with the biologic activating surface, thus interfering with the reciprocal activation between factor XII and prekallikrein.

SPR analysis also supports this assumption. Zn\(^{2+}\) ions affected the interaction between haemaphysalin and target molecules. Zn\(^{2+}\) ion-binding to factor XII/XIa and HK induces conformational changes in these molecules (12–14). Thus, haemaphysalin might bind to these molecules by recognizing their Zn\(^{2+}\) ion-induced conformational changes. Zn\(^{2+}\) ions, on the other hand, are necessary for the binding of factor XII and HK to the biologic activating surface, suggesting that putative receptors on the surface also recognize the Zn\(^{2+}\) ion-induced conformational changes of these molecules. Because putative receptors on the biologic activating surface are suggested to be common to factor XII and HK (15–17), these results suggest that haemaphysalin adheres to both contact factors by mimicking such common receptors.

Ixolaris is a Kunitz-type anticoagulant identified from the hard tick, *Ixodes scapularis*, that is assumed to interact with the exosite of factor X/Xa, not with the active site to factor Xa (24). The C-terminal Kunitz domain of ixolaris, which participates in this binding, contains only four Cys residues. Loss of Cys residues in this domain probably increases structural flexibility and

Figure 8: Sensorgrams for interaction between haemaphysalin and peptides derived from domain D5. Haemaphysalin was immobilized onto the sensorchip at a level of 1702 RU. (A) Three peptides (1 μM), HK383–420, HK421–466, and HK459–513, were injected onto the sensorchip at a flow rate of 20 μl/min. The interaction between haemaphysalin and HK421–466 was measured in the presence of 10 μM ZnCl\(_2\). Interactions between haemaphysalin and the other two peptides were monitored in the absence of Zn\(^{2+}\) ions. The sensorchip surface was regenerated by injection of 50 mM EDTA and 1 M NaCl. Interactions of haemaphysalin were measured with various concentrations of with HK421–466 (B) and HK459–513 (C). The assay procedure was similar to (A).
enables it to evolve to interact with sites that are different from the active site. Similar to ixolaris, haemaphysalin consists of two Kunitz-type protein domains, and their C-terminal domains lack two Cys residues. Haemaphysalin, therefore, might also have evolved to bind with cell-binding domains by lacking a disulfide bond in its C-terminal domain.

Haemaphysalin also prolonged PT. Our preliminary results suggest that haemaphysalin does not inhibit the amidolytic activity of factor VIIa-tissue factor, factor Xa, or thrombin, but inhibits generation of factor Xa in plasma (unpublished data). Thus, haemaphysalin might prolong PT by different mechanisms from typical Kunitz-type anticoagulants, as demonstrated by the prolongation of APTT.

In summary, the saliva of a hard tick, _H. longicornis_, contains a Kunitz-type inhibitor of the plasma kallikrein-kinin system and this inhibitor, haemaphysalin, acts by preventing reciprocal activation of factor XII and prekallikrein, the initial phase of activation of this system. We suggested that haemaphysalin recognizes Zn²⁺ ion-induced conformational changes of factor XII and HK, and prevents them from associating with the activating surface. Activation of the kallikrein-kinin system initiates acute-inflammatory reactions around the tick feeding sites and thereby enhances haemostatic reactions. Haemaphysalin might inhibit these host responses and facilitate blood sucking by the tick.

### References


31. De La Cadena RA, Colman RW. The sequence of _H. longicornis_, contains