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論　文　の　内　容　の　要　旨

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論文題目　Study on functional characterization of longistatin, a novel salivary gland protease, from the vector tick, *Haemaphysalis longicornis*
（フタトゲチマダニ新規唾液腺蛋白分解酵素ロンギスタチンの機能性状に関する研究）

Ticks (Arthropoda: Ixodoidia) are notorious hematophagous ectoparasites and serve as a unique vector of various deadly diseases inflicting humans and animals. Among the ticks, the ixodid ticks or hard ticks are more common and widely distributed throughout the world. Hard ticks feed on blood-meals for a long time (5−10 days or more) making a large blood pool, the essential feeding lesion of ticks, beneath the host's skin. Prior studies suggest that hard ticks produce a vast array of pharmacologically active biomolecules that are injected into the feeding lesions during persistent blood-feeding processes, and play crucial modulatory roles in their feeding success. However, the precise molecular mechanism(s) that prevents blood coagulation and initiates fibrinolysis in the blood pool to facilitate successful acquisition of blood-meals is still unclear. This study was performed to characterize a novel cDNA encoding an EF-hand protein from the salivary glands of the ixodid tick, *Haemaphysalis longicornis*, and the protein has been named as longistatin. Biochemical and functional characterization revealed that longistatin plays crucial roles in the development and maintenance of blood pool by keeping the blood in a fluid state through out the entire feeding period of the ticks; thus, longistatin is essential for blood-feeding success of hard ticks and eventually for the survival of ticks.

Chapter 1: Identification of an EF-hand protein from the salivary glands of the ixodid tick, *Haemaphysalis longicornis*

Longistatin gene was cloned from the salivary-gland cDNA libraries of adult *H. longicornis* ticks. Sequence analysis revealed that the full-length longistatin cDNA consisted of 750 bases. The open reading frame (ORF) consisting of 471 nucleotides, extends from the residues 133–603 which codes for a protein of 156 amino acid residues having a calculated molecular mass of 17,788 Da and a pI of 4.84. The molecule has a signal peptide and is predicted to be cleaved at Ala^{21}–Gln^{22}. The mature protein has a predicted molecular mass of 15,541 Da with a theoretical pI of 4.59. Longistatin contains two EF-hand Ca^{2+}-binding domains at residues 83–94 and 135–146 and the domains conserve canonical structures. Longistatin shows distinct changes in its migration during electrophoresis through SDS-PAGE gels containing calcium or ethylenediaminetetraacetic acid (EDTA). Longistatin moves faster in the presence of Ca^{2+}. Both recombinant and endogenous forms of longistatin can be stained with Rutheninum red, demonstrating that longistatin is a Ca^{2+}-binding protein. Reverse-transcription PCR data showed that *longistatin*-specific transcript was expressed in all lifecycle stages of *H. longicornis* and was up-regulated only in blood-fed ticks. Organ-specific transcription analysis revealed a salivary gland-specific expression of the gene which peaked at 96−120 h of feeding but declined sharply as soon as they dropped off the host. Consistently, endogenous longistatin was localized in the salivary glands of ticks and also in feeding lesions at the site of attachment of ticks on the host, suggesting that longistatin is synthesized in, and is secreted from, the salivary glands and may have functional roles in the feeding processes of ixodid ticks.

Chapter 2: Enzyme kinetics of longistatin

In this chapter, the enzyme kinetics of the purified recombinant longistatin was determined. Although longistatin does not contain the conserved catalytic triad of typical serine proteases but it efficiently hydrolyzed several serine protease-specific fluorogenic, synthetic substrates. Among the serine protease-specific substrates, longistatin potently hydrolyzed those containing Arg at the P1 site, indicating its specific affinity for the amide bond of Arg. Catalytic rate was relatively high during hydrolysis of α-thrombin-specific substrate (K_{Cat}/K_m 2.97 $M^{-1}s^{-1}$) followed by that of tissue-type plasminogen activator (t-PA)/urokinase-type plasminogen activator (u-PA) (K_{Cat}/K_m 2 $M^{-1}s^{-1}$) and trypsin-specific substrate (K_{Cat}/K_m 0.88 $M^{-1}s^{-1}$). Longistatin did not hydrolyze synthetic substrates specific for other groups of proteases. The enzyme was active at a wide range of temperature and pH, with the optimum at 37 °C and pH 7. Its activity was efficiently inhibited by various serine protease inhibitors such as,

phenylmethylsulfonyl fluoride (PMSF), aprotinin, antipain and leupeptin with the estimated IC_{50} of 278.57 µM, 0.35 µM, IC_{50} 41.56 µM and 198.86 µM, respectively. Additionally, longistatin was reacted with several cations; among them, $Ca^{2+}$and Mg^{2+} slightly increased the enzymatic activity of longistatin and Zn^{2+} potently inhibited longistatin in a concentration-dependent manner with an IC_{50} value of 275 µM. Mn^{2+} also attenuated its functions but to a much smaller extent. The inhibitory effect of Zn^{2+} was completely revived by EDTA. Given that, these findings suggest that longistatin is a new potent atypical serine protease.

Chapter 3: Longistatin activates plasminogen and degrades fibrinogen of hosts

In the chapter 3, biological functions of longistatin were evaluated. To explore the biological functions, longistatin was incubated with several commercially available proteins related with the feeding of blood-meals from hosts, such as fibrinogen, plasminogen, thrombin, factor VIIa and factor Xa. The study revealed that longistatin (1.6 µM) potently degraded the α, β and γ chains of fibrinogen as it was done by plasmin (1.6 µM), and delayed fibrin clot formation. Longistatin was shown to bind with fibrin with the estimated K_d, B_{max} and molar binding ratio (MBR) of 145.5±3.3 nmol/L, 3.1±0.6 µmol/L and 42.3±7.4, whereas those of t-PA were 159.2±7.4 nmol/L, 1.4±0.4 µmol/L and 19.3±4.7, respectively. Longistatin activated fibrin clot-bound plasminogen into its active form plasmin, as comparable to that of t-PA, and induced lysis of fibrin clot and platelet-rich thrombi. Longistatin induced more than 50% lysis of thrombi within 2 h at 640 nM concentration. Plasminogen activation potentiality of longistatin was increased up to 4 times by soluble fibrin. Taken together, these results suggest that longistatin may exert potent functions both as a plasminogen activator and as an anticoagulant in the complex scenario of blood pool formation.

Chapter 4: Longistatin is relatively resistant to plasminogen activator inhibitor-1

In this chapter, I have demonstrated that longistatin is resistant to Plasminogen activator inhibitor-1 (PAI-1) and activates plasminogen in the presence of PAI-1, and induces fibrinolysis. To determine the effect of platelet lysate on longistatin, I employed two-step indirect fluorogenic assays using plasmin-specific synthetic fluorogenic substrate (Boc-Glu-Lys-Lys-MCA). Longistatin was relatively less susceptible to the inhibitory effect of SDS-treated platelet lysate than physiologic PAs. Platelet lysate inhibited t-PA and two chain u-PA (tcu-PA) with the IC_{50} of 7.7 and 9.1 µg/ml, respectively, whereas for longistatin inhibition IC_{50} was 20.1 µg/ml ($p<0.01$). To explore the effect of PAI-1 on longistatin, I conducted direct fluorogenic assays using a t-PA-/u-PA-specific synthetic fluorogenic substrate

(Pyr-Gly-Arg-MCA) since longistatin also hydrolyzed this substrate. Activated PAI-1 (20 nM) inhibited only 21.47% activity of longistatin but almost completely inhibited t-PA (99.17%) and tcu-PA (96.84%). Interestingly, longistatin retained its 76.73% initial activity even after 3 h of incubation with 20 nM of PAI-1. IC_{50} of PAI-1 during longistatin inhibition was 88.3 nM while it was 3.9 and 3.2 nM in t-PA and tcu-PA inhibition, respectively. Longistatin completely hydrolyzed fibrin clot in the presence of 20 nM of PAI-1. Importantly, unlike t-PA, longistatin did not form complex with PAI-1, implying that longistatin is resistant to PAI-1 and may be an interesting tool for the development of a PAI-1 resistant effective thrombolytic agent.

Chapter 5: Effects of longistatin on blood feeding of ticks

To study the biological functions of longistatin directly in ticks, I conducted RNAi study by injecting *longistatin*-specific dsRNA into ticks through 4th coxa. All ticks microinjected with dsRNA were active and healthy during the incubation period. After placement on rabbit ears, all ticks of both treated and control groups actively attached. However, in the ds*longistatin*-injected group, 3 (2.5%) ticks were found dead at 72 h of attachment. All ticks in the ds*mal*E-injected group reached to repletion and detached by day 6 post-attachment. Notably, ds*longistatin* injection was shown to hamper the feeding of ticks. These ticks were poorly fed and most of them failed to engorge. Only two ticks (1.66%) dropped off the host following engorgement in the RNAi group. The mean body weight of the ticks collected after 6 days of feeding was significantly ($P<0.01$) lower in the RNAi-treated group (53.53±50.38 mg) than that of the control group (253.43±57.91 mg). A marked difference between blood pools induced by the ticks of RNAi-treated and control groups was observed. Large blood pools were developed at the site of attachment of each tick of the control group. Histologically, blood pools of control group were flooded with RBC but hemorrhagic changes were not detected at the biting areas of ticks of the RNAi-treated group, suggesting that the *longistatin* gene plays vital roles in the formation and maintenance of a blood pool as preceded by marked hemorrhage into tick-feeding lesions.

In conclusion, longistatin is synthesized in salivary glands, secreted with saliva and injected into host tissues during acquisition of blood-meals. Longistatin is essential for the development and maintenance of blood pool; thus, in the feeding processes and survival of hard ticks. Therefore, longistatin may be a potential target for the development of safe acaricide and effective anti-tick vaccine to control tick and tick-borne diseases.