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The role of HiTI, a serine protease inhibitor from *Haematobia irritans irritans* (Diptera: Muscidae) in the control of fly and bacterial proteases

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Abstract

Blood-sucking arthropods are vectors responsible for the transmission of several pathogens and parasites to vertebrate animals. The horn fly *Haematobia irritans irritans* (Diptera: Muscidae) and the tick *Boophilus microplus* are important hematophagous ectoparasites that cause losses in cattle production. A serine protease inhibitor from a thorax extract of the fly *H. irritans irritans* (HiTI) was previously isolated, characterized and cloned. In the present study we described the expression, purification, and characterization of the recombinant HiTI (rHiTI) and its possible role in the control of different endogenous and bacterial proteases. rHiTI was successfully expressed using the pPIC9 expression vector with a yield of 4.2 mg/L of active rHiTI. The recombinant HiTI purified by affinity chromatography on trypsin–Sepharose had a molecular mass of 6.53 kDa as determined by LS-ESI mass spectrometry and inhibition constants (Kis) similar to those of native HiTI for bovine trypsin and human neutrophil elastase of 0.4 and 1.0 nM, respectively. Purified rHiTI also showed inhibitory activity against the trypsin-like enzyme of *H. i. irritans* using its possible natural substrates, fibrinogen and hemoglobin; and also inhibited the OmpT endoprotease of *Escherichia coli* using fluorogenic substrates. The present results confirm that HiTI may play a role in the control of fly endogenous proteases but also suggest a role in the inhibition of pathogen proteases.

Keywords: Fly; Serine protease inhibitor; Innate immunity; Omp-T endoprotease

1. Introduction

The horn fly, *Haematobia irritans irritans*, is an important ectoparasite of cattle in tropical and sub-tropical countries (Cupp et al., 1998; Guerrero, 2000; Hori et al., 1981). Horn flies have been a problem in Brazil since their first appearance. This fly species is responsible for the transmission of the nematode *Stephanofilaria stiles* which damages hides (Hibler, 1966) and of *Staphylococcus aureus* which causes mastitis in dairy heifers (Owens et al., 1998). It was also suspected that *H. irritans irritans* is a vector of *Dermatobia hominis* in the Center West region of Brazil (Gomes et al., 1998). Heavy infestations with the horn fly cause intensive cattle irritation, with consequent considerable losses in meat and milk production (East et al., 1995; Elvin et al., 1993). The fly has been controlled with the use of insecticides, but is currently adapting to the chemical pressure and acquiring insecticide resistance (Byford et al., 1999; Sparks et al., 1990). Therefore, new methods have had been

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developed to control this insect, including a vaccine containing recombinant antigens (Cupp et al., 2004).

During the last decade, several researchers have studied this fly species mainly at the level of protein biochemistry and have reported the following activities: anti-hemostatic molecules (Cupp et al., 1998), serine proteases (Dametto et al., 2000; East et al., 1995), and serine protease inhibitors (Azzolini et al., 2004). The role of serine proteases of vertebrate animals in physiological processes such as digestion, blood clotting, embryogenesis, and immune response have been studied intensively. In invertebrate animals, serine proteases and their inhibitors have not been as well studied but are already known to be involved in some physiological processes (Simonet et al., 2002). Several Kunitz-type protease inhibitors have been described in arthropods, such as *Bombyx mori* (Sasaki and Kobayashi, 1984), *Manduca sexta* (Ramesh et al., 1988), and *Sarcophaga bullata* (Papayannopoulos and Biemann, 1992), most of them in the insect hemolymph. The role of these inhibitors is still unknown but it has been suggested that they may be involved in insect defense, inhibiting microbial proteases (Kanost, 1999).

In addition, the *Escherichia coli* outer membrane endoprotease (OMP-T), a serine protease which mainly cleaves between two consecutive basic residues (Dekker et al., 2001), has been suggested to be the major protective factor of *E. coli* against antimicrobial cationic peptides from the environment (Stumpe et al., 1998).

In a previous study, we described the purification, characterization, and cloning of HiTI, a serine protease inhibitor from the fly *H. irritans irritans*. This inhibitor belongs to the serine protease BPTI-Kunitz-type family and was shown to be a strong inhibitor of bovine trypsin, human neutrophil elastase and a trypsin-like enzyme from the same fly species (Azzolini et al., 2004). In the present paper, we describe the expression, purification and characterization of recombinant HiTI, and its possible role in the control of endogenous and bacterial serine proteases.

2. Materials and methods

2.1. Cloning and expression of the HiTI gene

The HiTi gene was amplified by PCR using the construction pHiTI 9.1 [1] and the primers HIPIC fwr 5′-CTCGAGAAAAGATTTGATAAAGCTGACTG-3′ and HIPIC rev 5′-ATA GTT TAG CGG CCG CTT ACA TGC ATG ACT GCT GCG ATT G-3′. The PCR product was digested with the *Xho*I and *Not*I restriction enzymes, purified, and ligated into the pPIC9 expression vector (Invitrogen, USA). The construction pSA 1.1.4 was used to transform *Pichia pastoris* GS115 (his4, Mut+) by electroporation. HiTI was expressed according to the manufacturer's instructions.

2.2. Purification of recombinant HiTI

Yeast was incubated for 5 days in BMMY medium. After fermentation, yeast cells were harvested by centrifugation (4000*g*, 20 min, 4 °C). The yeast supernatant was applied to a trypsin–Sepharose column previously equilibrated with 50 mM Tris–HCl buffer, pH 8.0. Weakly bound proteins were washed out with 50 mM Tris–HCl buffer, pH 8.0, containing 0.2 M NaCl, and the recombinant HiTI (rHiTI) was eluted with 0.2 M KCl, pH 2.0. The eluted fractions were immediately neutralized with 1 M Tris–HCl buffer, pH 8.0. The fractions containing the recombinant HiTI were pooled and stored at −20 °C.

2.3. Determination of apparent dissociation constant (K_i)

The equilibrium dissociation constants for complexes of rHiTI and bovine trypsin, and human neutrophil elastase were determined by the method of Morrison (1969). Briefly, the serine proteases were incubated with the inhibitor at different concentrations in 0.1 M Tris–HCl, pH 8.0, 0.15 M NaCl, and 0.1% Triton X-100 at 37 °C and the residual activities were measured after adding the following chromogenic substrates: Tosyl-Gly-Pro-Arg-pNA (for trypsin) and MeO-Suc-Ala-Ala-Pro-Val-pNA (for human neutrophil elastase). Apparent K_i values were calculated by fitting the steady state velocities to the equation describing the tight-binding inhibitor model using a non-linear regression analysis (Morrison, 1969).

2.4. SDS–polyacrylamide gel electrophoresis

The rHiTI was analyzed by SDS–PAGE using 15% acrylamide gels in Tris–glycine buffer by the method of Laemmli (1970). The gels were stained with Coomassie Blue R250.

2.5. Serine protease inhibition using natural substrates

Fibrinogen (Behring, Germany) and hemoglobin (Sigma, USA) digestion experiments were performed using 20 μg of each protein; *H. irritans irritans* trypsin-like enzyme (26 nM) and rHiTI (12 μM) were incubated in 10 mM Tris–HCl buffer, pH 8.0, for 30 min at 37 °C. After incubation, the samples were reduced with DTT (200 μg/ml) and heated at 96 °C for 10 min. Reduced samples were analyzed by SDS–PAGE.

2.6. N-terminal amino acid sequencing

Purified r-HiTI was applied to a C_8 Sephasil Peptide column (Amersham Biosciences, Sweden) and the