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Selective inhibitors of digestive enzymes from *Aedes aegypti* larvae identified by phage display

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ABSTRACT

Dengue is a serious disease transmitted by the mosquito *Aedes aegypti* during blood meal feeding. It is estimated that the dengue virus is transmitted to millions of individuals each year in tropical and subtropical areas. Dengue control strategies have been based on controlling the vector, *Ae. aegypti*, using insecticide, but the emergence of resistance poses new challenges. The aim of this study was the identification of specific protease inhibitors of the digestive enzymes from *Ae. aegypti* larvae, which may serve as a prospective alternative biocontrol method. High affinity protein inhibitors were selected by all of the digestive serine proteases of the 4th instar larval midgut, and the specificity of these inhibitors was characterized. These inhibitors were obtained from a phage library displaying variants of HiTI, a trypsin inhibitor from *Haematobia irritans*, that are mutated in the reactive loop (P1–P4′). Based on the selected amino acid sequence pattern, seven HiTI inhibitor variants were cloned, expressed and purified. The results indicate that the HiTI variants named T6 (RGGAV) and T128 (WNEGL) were selected by larval trypsin-like (IC_{50} of 1.1 nM) and chymotrypsin-like enzymes (IC_{50} of 11.6 nM), respectively. The variants T23 (LLGGL) and T149 (GGVWR) inhibited both larval chymotrypsin-like (IC_{50} of 4.2 nM and 29.0 nM, respectively) and elastase-like enzymes (IC_{50} of 1.2 nM for both). Specific inhibitors were successfully obtained for the digestive enzymes of *Ae. aegypti* larvae by phage display. Our data also strongly suggest the presence of elastase-like enzymes in *Ae. aegypti* larvae. The HiTI variants T6 and T23 are good candidates for the development as a larvicide to control the vector.

1. Introduction

Mosquitoes are important vectors of diseases such as malaria, filariasis, dengue and yellow fever (Paul et al., 2006). *Aedes aegypti* (Diptera: Culicidae) is the major vector of dengue viruses, which are responsible for more human mortality and morbidity than any other arthropod-transmitted viral disease (Alphey et al., 2002). In recent decades, dengue prevalence has grown dramatically around the world. Over 2.5 billion people (~40%) are at risk of infection (WHO, 2012). The WHO estimates new dengue cases at 50–100 million every year worldwide. Across the Americas, Southeast Asia and Western Pacific, dengue cases exceeded 1.2 million in 2008 and over 2.2 million in 2010. Of these, 1.6 million cases were reported in the Americas alone of which 49,000 cases were severe dengue (WHO, 2012).

Serine proteases such as trypsin and chymotrypsin play various important roles in food digestion, immune defense and zymogen activation in insects (Ge et al., 2012). In mosquitoes, the digestive enzymes are primarily serine proteases (Lopes et al., 2004). Some digestive enzymes have been identified and characterized in *Ae. aegypti* adults, including trypsin (Barillas-Mury and Wells, 1993), chymotrypsin (Jiang and Kanost, 1997), aminopeptidase (Morlais et al., 2003) and carboxypeptidases (Isoe et al., 2009). Despite several studies concerning adult *Ae. aegypti* digestive biochemistry and molecular biology, very few studies have been performed to elucidate the digestion in *Ae. aegypti* larvae. The few studies conducted on the digestion in larvae have focused on the description and identification of midgut digestive enzymes. Trypsin-like and chymotrypsin-like activities have been described in *Ae. aegypti* larvae (Yang and Davies, 1971; Ho et al., 1992; Borovsky and Meola, 2004; Mesquita-Rodrigues et al., 2011). It was demonstrated that

Abbreviations: *Ae. aegypti*, *Aedes aegypti*; HiTI, *Haematobia irritans* Trypsin Inhibitor; CFU, Colony Forming Unit.

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the amount of trypsins is twice as high as that of chymotrypsins in the 4th instar larvae of *Ae. aegypti* (Borovsky and Meola, 2004). Recently, we performed a transcriptional analysis of trypsin-like enzymes present in the *Ae. aegypti* larval midgut and purified the major native trypsin-like enzyme present in this organ (Soares et al., 2011). Due to the small size of mosquito larva and the large number of digestive proteins, the purification of all digestive proteases present in the larval midgut was not possible. Mosquito larvae are aquatic and feed constantly; therefore, the inhibition of digestive enzymes present in this life stage represents a potentially successful strategy to control mosquito populations.

Protease inhibitors, when added to the diet of insects, interfere with their digestive process by decreasing the assimilation of nutrients, leading to delayed development and mortality (Sales et al., 2000; Rodrigues Macedo et al., 2003; Napoleao et al., 2012). Molecular biology techniques have been used to select specific protease inhibitors, among which the phage display system may be viewed as a powerful method.

Phage display has proven to be a powerful molecular technique for selecting proteins or peptides with desired biological properties (Zani and Moreau, 2010). An enormous number of protease inhibitor scaffolds have been displayed at the surface of filamentous phages to select new inhibitors with altered specificities and enhanced affinities toward one or more target protease (Zani and Moreau, 2010). This technique was also used to select protease inhibitors for anticancer therapy (Zhan et al., 2012; Lu et al., 2012) and an inhibitor in the treatment of neurodegenerative diseases (Popiel et al., 2011).

Previously, our group has used the phage display system (phagemid pCANTAB 5E) to study and select specific inhibitors, such as chicken cystatin, a cysteine protease inhibitor (Tanaka et al., 1995); the leech-derived tryptase inhibitor (LDTI) (Campos et al., 2004; Tanaka et al., 1999a); and infestin 4, an inhibitor of *Triatoma infestans* that is specific for coagulation enzymes (Campos et al., 2012). Recently, a peptide display library was constructed to study substrate specificity for a cathepsin L-like enzyme (BmCL1) that is a cysteine protease of *Boophilus microplus* (Clara et al., 2011). In addition, an HiTI random library (with P1 = Arg) was constructed to select specific inhibitors of cuticle-degrading proteases (Pr2) of *Metarhizium anisopliae* fungus and bovine trypsin as a control (de Marco et al., 2010). In this study, a new library based on the identical inhibitor, HiTI, was constructed and subjected to selection by the digestive enzymes of *Ae. aegypti* larvae. Specific inhibitors were selected by *Ae. aegypti* larval midgut enzymes using this phage display method. The inhibitors that were selected strongly suggested that in addition to trypsin- and chymotrypsin-like enzymes, an elastase-like enzyme was also present in the larval midgut. At least two selected inhibitors may be suitable for the development of a larvicide to control the vector.

2. Materials and methods

2.1. Construction of the HiTI mutant library (P1–P4′)

The phagemid containing the HiTI gene was obtained from de Marco et al. (2010). The HiTI mutant DNA fragments were obtained by a PCR reaction using an oligonucleotide (Hidegfw) containing the degenerations at positions P1–P4′ and a reverse oligonucleotide (PCTBHIRV) with the construction phagemid-HiTI (de Marco et al., 2010) used as a template. The sequences of the oligonucleotides used were Hidegfw, 5′-AAAGAGGTTGGGCCCT-GTNNSNNSNNSNNSNNSTCGTACTACTATGATAC-3′, and PCTBHIRV, 5′-GCGAATTAATTCGCGGCGGCCGCCATGCATGACTGC-3′. The amplified mutated DNA fragments and the vector containing the HiTI fragment were digested by *Apa*I and *Not*I restriction enzymes. The mutated DNA fragments were ligated into the dephosphorylated phagemid pCANTAB 5E. Six ligations were performed using a ratio of 1:50 (vector:insert) and incubated overnight at 16 °C. Subsequently, all of the ligations were precipitated with a solution of ethanol and sodium acetate, and the DNA pellet was washed with 70% ethanol and dried at room temperature. The DNA precipitate was dissolved in sterile water (5 μL). The ligation products were used to perform six independent transformations of *E. coli* TG1 cells. The transformed cells were incubated in SOC medium with shaking at 150 rpm for 1 h at 37 °C. After incubation, all of the transformed cells were pooled into one fraction (library) that was amplified by inoculation in 200 mL of SOBAG medium containing glucose (2%), $MgCl_2$ (10 mM) and ampicillin (200 μg/mL) and reincubated with shaking at 200 rpm overnight at 30 °C. The amplified library was separated into aliquots containing 15% glycerol and stored at −70 °C. An aliquot of the original library was used in a library titration in which the cell suspension was diluted to 10^2, 10^3 and 10^4 with 2xYT medium. Then, 100 μL of each dilution were plated on SOBAG medium containing ampicillin (200 μg/mL) and incubated overnight at 30 °C. The number of colonies on each plate was determined, and the total quantity of library transformants was calculated. The amplified library was also titrated as described above except the cell suspension was diluted to 10^6, 10^7 and 10^8 in 2xYT medium.

2.2. Selection of HiTI variants by the digestive enzymes present in the midgut of 4th instar larvae from Ae. aegypti

The phage inhibitor selection was performed as previously described by Tanaka et al. (1999a). *E. coli* TG1 cells containing the phagemids were grown at 30 °C in 2xYT medium containing 200 μg/mL of ampicillin and 2% (w/v) glucose. Cells were cultured to an optical density (550 nm) between 0.5 and 0.7. Afterwards, helper phages (M13KO7) with a multiplicity of infection (MOI) of 100 were added to promote bacterial infection. The mixture was incubated with shaking (200 rpm) for 1 h at 37 °C and centrifuged. Subsequently, the supernatant was discarded, and the bacterial pellet was resuspended in fresh 2xYT medium containing 200 μg/mL of ampicillin and 50 μg/mL of kanamycin. The culture was grown with shaking (200 rpm) for 12–15 h at 37 °C. The bacterial culture was centrifuged, the pellet was discarded and the supernatant containing fusion phage particles was used for the selection of inhibitors by affinity binding to the crude extract of the 4th instar larval midgut from *Ae. aegypti*. A microplate (96 wells) was sensitized with 200 μL of enzyme solution (100 μL of midgut extract diluted two fold in 0.05 M sodium acetate buffer, pH 5.5, containing 0.1 M NaCl) for 18 h at 4 °C. The enzyme solution was removed, and the plate was blocked with 200 μL of blocking buffer (2% bovine albumin in PBS [6.6 mM sodium phosphate, 2.7 mM KCl, pH 7.4]) containing 0.005% Tween 20 for 2 h at 37 °C. In parallel, 200 μL of fusion phage particles were incubated with 200 μL of blocking buffer for 30 min at 37 °C. Afterwards, the blocking buffer was removed, and 100 μL of the fusion phage solution were added and incubated for 2 h at 37 °C. After the incubation with phage solution, the plate was washed exhaustively with PBS (6.6 mM sodium phosphate, 2.7 mM KCl, pH 7.4) containing 0.1% Tween 20 and then with 0.2 M KCl solution, pH 5.0. After this step, phages were eluted with 400 μL of 0.2 M KCl solution, pH 2.0, and neutralized with 10 μL of 1.0 M Tris–HCl buffer, pH 8.0. The eluted phages were titered and used to re-infect *E. coli* TG1 cells (400 μL of TG1 cells with 400 μL of phages) for 30 min at 37 °C. Subsequently, the medium was replaced with 2xYT containing 200 μg/mL ampicillin and 2% (w/v) glucose, incubated overnight at 30 °C, and recovered using M13K07 helper phage (Pharmacia), as described above. After the third round of selection, tight-binding fusion phages were