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TO17: A teleost antimicrobial peptide that induces degradation of bacterial nucleic acids and inhibits bacterial infection in red drum, *Sciaenops ocellatus*

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ABSTRACT

Tissue factor pathway inhibitor (TFPI)-1 is well known for its role as an inhibitor of blood coagulation. Several studies have demonstrated that the C-terminal peptides of TFPI-1 are active against a broad spectrum of microorganisms. In a previous study, we found that TO17 (with 17 amino acids), a TFPI-1 C-terminal peptide from red drum (*Sciaenops ocellatus*), was active against *Edwardsiella tarda*. In the present study, we investigated further the antimicrobial spectrum, action mode, as well as the immunostimulatory property of TO17. Our results showed that TO17 displayed antimicrobial activity against *Staphylococcus aureus*, *Micrococcus luteus*, *Vibrio vulnificus*, and infectious spleen and kidney necrosis virus, independent of host serum. Furthermore, the activity of TO17 was influenced by the length or type of amino acids at the N and C termini. During its interaction with *V. vulnificus*, TO17 exerted its antibacterial activity by destroying cell membrane integrity, penetrating the cytoplasm and inducing degradation of genomic DNA and total RNA. In addition, TO17 had no hemolytic activity against red drum blood cells. *In vitro*, TO17 enhanced production of nitric oxide and bactericidal activity of red drum macrophages. *In vivo*, administration of red drum with TO17 before bacterial infection significantly reduced pathogen dissemination and replication in tissues. These results indicate that TO17 is a broad-spectrum antimicrobial peptide with immunostimulatory properties and it has the potential to be used as an antimicrobial agent in aquaculture.

1. Introduction

Outbreaks of infectious diseases are increasing in aquaculture, but their control still depends on traditional antibiotics in most areas of the world [1–5]. Overuse of antibiotics has serious side effects, such as drug residues in aquatic animals and the environment, and more seriously, the rapid emergence of diverse multidrug-resistant strains of bacterial pathogens [6,7]. This phenomenon has prompted a sustained search for new antimicrobial agents that act in a different way from traditional antibiotics [8]. Polycationic antimicrobial peptides (AMPs) are present in virtually all organisms as part of the innate immune system and they act as endogenous antibiotics [9]. These peptides could induce the direct destruction of a wide diversity of microorganisms [9,10]. Due to their ability to attack different microorganisms, including bacteria, viruses and fungi, and without the development of resistance, AMPs have been regarded as promising candidates for the development of novel antibiotics [11].

Tissue factor pathway inhibitor (TFPI)-1 is a matrix-associated Kunitz-type serine protease inhibitor, known for its ability to inhibit blood coagulation [12]. In structure, TFPI-1 is composed of a highly negatively charged N-terminus, three bovine pancreatic trypsin inhibitor (BPTI)/Kunitz domains, and a highly positively charged C terminus [13–15]. The C-terminal region is required for interactions with factor Xa, heparin, and some types of cells. The C-terminal region of human TFPI-1 can be cleaved off at different positions, resulting in various truncates and C-terminal fragments, which display antimicrobial activity against pathogenic bacteria and fungi [16]. In teleosts, although several TFPI-1 molecules have been identified, the study of biological functions of TFPI-1 have been limited to red drum (*Sciaenops ocellatus*) and tongue sole (*Cynoglossus semilaevis*). In red drum, the C-terminal peptide of TFPI-1 possesses greater antibacterial activity than recombinant TFPI-1 [17]; in tongue sole, the C-terminal peptide of TFPI-1 displays broad-spectrum activity [18].

Red drum was discovered originally in the Atlantic Ocean and the Gulf of Mexico; it was introduced into China in 1991 and since then it has been cultured extensively in several provinces in China [17]. However, the red drum industry has long been suffering from serious diseases and there are still no effective prevention and control measures. In a previous study, we reported that TO17, a peptide derived from the C terminus of red drum TFPI-1, possesses antibacterial activity

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against *Edwardsiella tarda* [17]. In this study, we examined further the antimicrobial spectrum and mode of action of TO17, and investigated the immunostimulatory properties and potential use in fighting invasive pathogens.

2. Materials and methods

2.1. Fish

Clinically healthy red drum (average 10.3 ± 1.7 g) were purchased from a commercial fish farm in Fujian Province, China and maintained at 25 °C in aerated seawater. Before experimental manipulation, fish were acclimatized in the laboratory for 2 weeks and randomly sampled for the presence of bacteria and viruses as reported previously [19,20]. Fish without pathogens were selected for experimental use, and killed with an overdose of tricaine methanesulfonate (Sigma, St. Louis, MO, USA) prior to experiments involving tissue collection [21].

2.2. Bacterial strains and culture conditions

The fish pathogens *Klebsiellar pneumonia* J1, *Pseudomonas putida* C1, *Serratia marcescens* X1, *Streptococcus agalactiae* G1, *Vibrio anguillarum* CJ, *Vibrio harveyi* Z1, *Vibrio litoralis* H1 *Vibrio parahaemolyticus* PL2 and *Vibrio scophthalmi* ZS1 were preserved in our laboratory. *Escherichia coli* DH5α was purchased from Tiangen (Beijing, China), and *Micrococcus luteus* 1D00051, *Staphylococcus aureus* 1D00101 and *Vibrio vulnificus* 1H00066 were purchased from China General Microbiological Culture Collection Center (Beijing, China). Except for *S. agalactiae* G1, which was cultured in Brain Heart Infusion broth, all strains were cultured in Luria–Bertani (LB) medium at 37 °C (for *E. coli*, *M. luteus* and *S. aureus*) or 28 °C (for all others). Fish megalocytivirus and infectious spleen and kidney necrosis virus (ISKNV) were kindly provided by Doctor Li of Pearl River Fishery Research Institute, Chinese Academy of Fishery Sciences, and was propagated in a continuous cell line (named as CPB) established previously from brain of *Siniperca chuatsi* [22].

2.3. Peptides

Unlabeled TO17 [17], CTO17 (CKCRRRKVHGPMIRIRKK), TO15 (KCRRRKVHGPMIRIR), TO19 (KCRRRKVHGPMIRIRKKNL), TO23 (KCRRRKVHGPMIRI RKKNLDNIL) and control peptide P86P15 [23]; 5′-FITC labeled TO17 and P86P15 were chemically synthesized by Pepmic (Suzhou, China). These peptides were purified by high-performance liquid chromatography to 95% purity. Lyophilized peptides were stored at −20 °C and dissolved in phosphate-buffered saline (PBS, pH 7.4) before use.

2.4. Assay of antibacterial spectrum

Antibacterial spectrum assay was carried out as previously reported [18]. The bacteria were cultured to mid-logarithmic phase. The cells were centrifuged, washed, and resuspended in PBS to 2×10^6 CFU/ml. 50 μL of the suspension was plated on LB agar plates, the sterile filter papers were slipped onto the LB plates, and 5 μL TO17 (300 μM) was added to the filter paper. All plates were cultured as above for 24 h, and the antibacterial effect was determined according to the presence of an inhibition zone.

2.5. Assay of antiviral activity

ISKNV (10^5 copies/ml) was mixed with TO17 or P86P15 at a final concentration of 140 μM. PBS was used as the negative control. The mixture was incubated at 25 °C for 4 h, after which the fish were inoculated by intraperitoneal (i.p.) injection of 100 μL of the mixture. Spleens were taken under aseptic conditions at 3, 5 and 7 d post-infection. Viral copy numbers in the spleens were determined by absolute quantitative real-time polymerase chain reaction, as reported previously with primers ISKNV MCP RTF1 (5′-GCGAGTTCCTTGACTTC TGG-3′) and ISKNV MCP RTR1 (5′- CCTGGTTGCTCTGGCTGAT-3′) [24].

2.6. Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) assays

The MIC assay was performed as previously reported [18]. The target bacteria were cultured as described above to mid-logarithmic phase. The cells were centrifuged, washed, and resuspended in PBS to 2×10^6 CFU/ml. TO17, CTO17, TO15, TO19, TO23 and P86P15 were dissolved in PBS and diluted serially twofold. The dilution (1 μL each) was mixed with 10 μL bacterial suspension and 39 μL LB. For MIC assay, the mixture was incubated at 28 or 37 °C as above for 24 h and then inspected for growth. MIC was defined as the lowest peptide concentration that prevented visible growth. For the MBC assay, the mixture was incubated at 28 °C or 37 °C for 24 h; after incubation, the mixture was diluted and plated in triplicate on LB agar plates. The plates were incubated at 28 °C or 37 °C for 48 h, and the colonies growing on the plates were counted. MBC was defined as the lowest peptide concentration that resulted in no colony emergence on the plates. The assays were performed three times.

2.7. Serum and blood cells preparation and assays

Serum and blood cells were collected from red drum. The serum was frozen in aliquots at −80 °C and the specimens were thawed quickly to retain complement activity and used within 15 min. In bactericidal assays, sera were added at a ratio of 20% and bacterial survival rates were analyzed as described above. Whole blood was centrifuged at 800 g for 10 min, and plasma and buffy coat were removed. The erythrocytes were washed three times and resuspended in PBS to obtain a 5% suspension. The cells were added at a ratio of 1%, the sterile filter papers were slipped into the LB agar plates, and 5 μL TO17 (300 μM) was added to the filter paper. All plates were cultured as above overnight, and the hemolysis effect was measured.

2.8. Killing kinetics

Dose–killing assays were performed as previously reported [18]. Suspensions of target bacteria were prepared as described above. The cells were centrifuged, washed, and resuspended in PBS to 2×10^6 CFU/ml. The target bacteria were incubated with different concentrations of TO17 (range from 0 to 100 μM) for 3 and 6 h. After incubation, aliquots were taken and diluted appropriately in PBS, and 50-μl aliquots were plated in triplicate on LB agar plates. The inoculated plates were incubated for 24 h before colony counting.

2.9. Transmission electron microscopy

Transmission Electron Microscopy (TEM) was performed as reported previously [18]. The target bacterium *V. vulnificus* was cultured in LB agar plates to mid-logarithmic phase and resuspended in PBS to 2×10^6 CFU/ml. One hundred microliters of bacterial cells were treated with 0.5 × MIC, 1.0 × MIC and 2.5 × MIC TO17 at 28 °C for 3 h. After incubation, the cells were fixed with glutaraldehyde and deposited on carbon-coated copper grids. The grids were dried naturally and negatively stained with phosphotungstic acid. The grids were then observed with TEM(GEM-1200, Jeol, Japan).

2.10. Fluorescence microscopy

Fluorescence microscopy was performed as reported previously [18]. *V. vulnificus* was cultured as above and resuspended in PBS to 2×10^6 CFU/ml. Twenty microliters of bacterial cells were incubated