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A TFPI-1 peptide that induces degradation of bacterial nucleic acids, and inhibits bacterial and viral infection in half-smooth tongue sole, *Cynoglossus semilaevis*

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ABSTRACT

Tissue factor pathway inhibitor 1 (TFPI-1) is a serine protease inhibitor that inhibits tissue factor (TF)-mediated coagulation. The C-terminal region of TFPI-1 could be cleaved off and proved to be antimicrobial against a broad-spectrum of microorganism. In a previous study, a C-terminal peptide, TC24 (with 24 amino acids), derived from tongue sole (*Cynoglossus semilaevis*) TFPI-1, was synthesized and found antibacterial against *Micrococcus luteus*. In the present study, the antibacterial spectrum and the action mode of TC24 was further examined, and its *in vivo* function was analyzed. Our results showed that TC24 also possesses bactericidal activity against *Staphylococcus aureus* and *Vibrio vulnificus*. During its interaction with the target bacterial cells, TC24 destroyed cell membrane integrity, penetrated into the cytoplasm, and induced degradation of genomic DNA and total RNA. *In vivo* study showed that administration of tongue sole with TC24 before bacterial and viral infection significantly reduced pathogen dissemination and replication in tissues. These results indicated that TC24 is a novel antimicrobial peptide against bacterial and viral pathogens, and that the observed effect of TC24 on bacterial RNA adds new insights to the action mechanism of fish antimicrobial peptides. Moreover, TC24 may play an important role in fighting pathogenic infection in aquaculture.

1. Introduction

In aquaculture, the control of diseases was still mainly depended on traditional antibiotics in most area of the world. The abuse of antibiotics caused serious side effects, such as the drug residue in aquatic animals and environment as well as the emergence of diverse multidrug-resistant bacterial strains [1–3]. Nowadays, the new antimicrobial substances that have the potential to replace the use of antibiotics were antimicrobial peptides (AMPs), which have attracted more and more interests. AMPs are short, cationic peptides that distributed ubiquitously from microorganisms, plants to animals [4–8]. During the antimicrobial process, AMPs directly kill the target microbe without the need of interaction with specific receptors [9,13], the unique action mode was different from that of antibiotics and prevents the development of resistant variants [9–13].

Tissue factor pathway inhibitor 1 (TFPI-1) is a matrix-associated Kunitz-type serine protease inhibitor that inhibits tissue factor (TF)-mediated coagulation activation [14]. Structurally, TFPI-1 consists of a highly negatively charged N terminus, three tandem Kunitz domains, and a highly positively charged C-terminus [15–17]. Previous reports have shown that the C-terminal region of TFPI-1 is required for interactions with plasma lipoproteins, thrombospondin-1, clearance receptors [18], and lipopolysaccharide [19]. The C-terminal region of human TFPI-1 could be cleaved off by a number of proteinases such as thrombin, plasmin, and matrix metalloptoteinase-8 at different positions *in vivo* and *in vitro*, resulting in various TFPI-1 and TFPI-1 C-terminally truncated forms [20–27]. These C-terminal peptides of human TFPI-1displayed antimicrobial activity against both pathogenic bacteria and fungi [28].

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In teleost, although a number of TFPI-1 molecules have been identified, but the study of TFPI-1 peptide was limited to red drum, *Sciaenops ocellatus*. In which, Zhang found the C-terminal peptide of TFPI-1 possess antibacterial activity higher than recombinant TFPI-1 [29], but the antibacterial mechanism of the peptide was unknown. The function of TFPI-1 peptide in other fish species still remain unknown.

Half-smooth tongue sole (*Cynoglossus semilaevis*) is a flat fish cultured widely in China as an economic species. However, tongue sole industry is long-suffering from serious diseases and there still no effective measure for control of these diseases. In a previous study [30], we have reported TC24, a peptide derived from C-terminal fragment of tongue sole TFPI-1, was antibacterial against *Micrococcus luteus*. In this study, we will further examine the antimicrobial spectrum and the action mode of TC24, as well as investigate the potential of TC24 in fighting pathogens invasion.

2. Materials and methods

2.1. Ethics statement

The experiments involving live animals were conducted in accordance with the "Regulations for the Administration of Affairs Concerning Experimental Animals" promulgated by the State Science and Technology Commission of Shandong Province. The study was approved by the ethics committee of Qingdao Agricultural University.

2.2. Fish

Clinically healthy half-smooth tongue sole (average 20.7 ± 1.1 g) were purchased from a commercial fish farm in Shandong Province, China and maintained at 20 °C in aerated seawater. Before experimental manipulation, fish were randomly sampled for the examination of the presence of bacteria and megalocytivirus in blood, liver, kidney, and spleen by bacteria colony counting or quantitative real time PCR (Polymerase Chain Reaction) as reported previously [31,32], and no pathogens were detected in these tissues. Fishes were euthanized with an overdose of tricaine methanesulfonate (Sigma, St. Louis, MO, USA) prior to experiments involving tissue collection [33].

2.3. Bacterial strains and culture conditions

The fish pathogens *Vibrio harveyi* Z1, *Serratia marcescens* ZX1 and *Klebsiella pneumonia* M1 were isolated recently from diseased fish and preserved in our laboratory. *Micrococcus luteus* 1D0005, *Staphylococcus aureus* 1D00101 and *Vibrio vulnificus* 1H00066 were purchased from China General Microbiological Culture Collection Center (Beijing, China). All strains were cultured in Luria-Bertani (LB) medium and cultured at 37 °C (for *M. luteus* and *S. aureus*) or 28 °C (for all others). Fish megalocytivirus, 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* [34].

2.4. Peptides

Unlabeled and 5′–FITC labeled TC24 [30] as well as control peptide P86P15, which corresponds to residues 22–36 of P86, a capsid protein of rock bream iridovirus isolate 1 from China (RBIV-C1) [35] were chemically synthesized by Pepmic (Suzhou, China). The peptides were purified by high-performance liquid chromatography to 90% of purity. Lyophilized peptides were stored at −20 °C and dissolved in Phosphate Buffered Saline (PBS, pH 7.4) before use.

2.5. Screening of antimicrobial spectrum

Above mentioned bacteria were cultured to mid-logarithmic phase. The cells were centrifuged, washed, and resuspended in PBS to 2×10^6 CFU/ml. 50 μl bacterial culture was plated on LB plate, the sterile filter papers were slipped into the LB plate and 5 μl of TC24 or P86P15 was added onto the filter paper. All plates were cultured as above for 24 h, and the antibacterial effect was determined according to the presence of inhibition zone.

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

The MIC and MBC of TC24 were determined according to the method previously reported [33]. Briefly, the target bacteria were cultured as above to mid-logarithmic phase. The cells were centrifuged, washed, and resuspended in PBS to 2×10^6 CFU/ml. TC24 or P86P15 were diluted serially in two-fold. The dilution (1 μl each) was mixed with 10 μl of bacterial suspension and 39 μl LB. For MIC assay, the mixture was incubated at 28 °C or 37 °C (as described above for different bacteria) for 24 h and then inspected for growth. MIC was defined as the lowest peptide concentration that prevented visible growth. For 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 that appeared 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 for three times.

2.7. Serum preparation and assays

Sera were collected from tongue sole and frozen in aliquots at −80 °C. The serum 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.

2.8. Killing kinetics

Target bacteria was cultured as described above. The cells were centrifuged, washed, and resuspended in PBS to 2×10^6 CFU/ml. The killing kinetics assay was measured according to the reported method with a little modification [36]. Briefly, the target bacteria were incubated with different concentrations of peptide determined by the above MIC assays. The viable counts were monitored up to 24 h. Aliquots were taken at defined intervals and diluted appropriately in PBS (pH 7.4), and then 50 μl of the dilutions were plated in triplicate on LB agar plates. The plates were incubated at 28 °C or 37 °C for 24 h, then the colonies that appeared on the plates were counted.

2.9. Electron microscopy

The target bacteria *M. luteus* and *V. vulnificus* were cultured in LB medium to mid-logarithmic phase and resuspended in PBS to 2×10^6 CFU/ml. One hundred microliters of bacterial cells were treated with 300 μM TC24 at 28 °C for 2 h, 4 h and 6 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 a transmission electron microscope (TEM) (GEM-

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