Fish & Shellfish Immunology 55 (2016) 662–670

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Synthetic hepcidin from fish: Uptake and protection against *Vibrio anguillarum* in sea bass (*Dicentrarchus labrax*)

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ARTICLE INFO

Article history:
Received 26 January 2016
Received in revised form
15 June 2016
Accepted 22 June 2016
Available online 29 June 2016

Keywords:
Hepcidin
Vibrio anguillarum
Sea bass
Peptide

ABSTRACT

The generation of a variety of new therapeutic agents to control and reduce the effects of pathogen in aquaculture is urgently needed. The antimicrobial peptides (AMPs) are one of the major components of the innate defenses and typically have broad-spectrum antimicrobial activity. However, absorption and distributions of exogenous AMPs for therapeutics application on farmed fish species need to be studied. Previous studies in our laboratory have shown the properties of hepcidin as an effective antimicrobial peptide produced in fish in response to LPS and iron. Therefore, we decided to investigate the antimicrobial activity of four synthetic variants of hepcidin against *Vibrio anguillarum in vitro*, and using the more effective peptide we demonstrated the pathogen's ability to protect against the infection in European Sea bass. Additionally the uptake of this peptide after ip injection was demonstrated, reaching its distribution organs such as intestine, head kidney, spleen and liver. The synthetic peptide did not show cytotoxic effects and significantly reduced the accumulated mortalities percentage (23.5%) compared to the European Sea bass control (72.5%) at day 21. In conclusion, synthetic hepcidin shows antimicrobial activity against *V. anguillarum* and the *in vivo* experiments suggest that synthetic hepcidin was distributed trough the different organs in the fish. Thus, synthetic hepcidin antimicrobial peptide could have high potential for therapeutic application in farmed fish species.

1. Introduction

Antimicrobial peptides (AMPs) are effector molecules present in the innate immune system of different multicellular organisms [1]. They are usually made up of 12–50 amino acid residues with hydrophobic regions and a net positive charge of +2 to +7 [2]. These chemical characteristics are relevant for the various modes of action described for these molecules, such as bacterial membrane rupturing by promoting the formation of pores or the binding to intracellular targets [3]. Among the variety of AMPs, cysteine-rich outstand because they can form disulfide bonds, stabilizing secondary structures and considerably increasing their antimicrobial properties [4]. One of the members of this family of peptides is hepcidin, also known as "Liver-Expressed Antimicrobial Protein (LEAP-1)" in humans, which contains eight cysteines and has a hairpin β-sheet type structure [5]. It is a type II acute-phase protein and has been proposed as a key mediator of anemia and inflammation [6].

Over the last decade, many antimicrobial peptides described in higher vertebrates have also been found in teleost fish. Some have been reported to be present in the mucous, suggesting they are part of primary defense against infectious agents, and as in higher vertebrates, have an essential role in innate immune response [7,8]. The hepcidin transcript has been identified as well as its expression in different teleost fish, as for example the Nile tilapia (*Oreochromis niloticus*), the Atlantic cod (*Gadus morhua*), the marine medaka

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http://dx.doi.org/10.1016/j.fsi.2016.06.035
1050-4648/

(*Oryzias melastigma*), the orange-spotted grouper (*Epinephelus coioides*), the Atlantic salmon (*Salmo salar*), the rainbow trout (*Oncorhynchus mykiss*), and the European seabass (*Dicentrarchus labrax*) [9–12]. Surprisingly, the eight cysteines are preserved in all these species, which emphasizes the relevance these residues have in the activity and configuration adopted by this peptide. A study carried out with the hepcidin of the hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) revealed it adopts a conformation similar to that of human hepcidin [13]. Moreover, our group has described the conformational change of hepcidin from the rainbow trout after the oxidation of its cysteines. The peptide changes to an alpha-helix conformation when in the reduced state, while it shows a β-sheet structure when in the oxidized state through the formation of disulfide bonds [14]. Furthermore, we have shown the presence of this peptide in trout liver and anterior kidney, and that it is upregulated in response to *Escherichia coli* or *Aeromonas salmonicida* lipopolysaccharide [15,16]. These results suggest that it participates in cellular defense responses of teleost fish against pathogen-associated molecular patterns (PAMPs), possibly being involved in post-invasion by the infectious agents [17].

Hepcidin, as other AMPs, has a wide spectrum of action. It has been described to be active against Gram-negative and Gram-positive bacteria. Furthermore, it can inhibit the growth of fungi and yeast and even has antiviral and immunomodulatory activity [13,18–20]. The properties of these peptides have caught the attention for their possible therapeutic use, particularly in production fields were antibiotics are routinely used to help prevent the occurrence of infections in aquaculture. In addition, the use of such antibiotics presents a number of problems associated with their incomplete biodegradability and consequently this has led to environmental problems and has had an influence on antimicrobial resistance in fish pathogens. Therefore, addressing the need of innocuous antimicrobial agents is essential in aquaculture [21,22]. It is interesting to study the antimicrobial properties of highly stable AMPs, such as hepcidin, to help fight recurrent pathogens in fish farms as an alternative to the existing chemical compounds involving treatment with a low dosage [23].

A previous study has addressed the possible antimicrobial use of hepcidin against pathogens present in fish farms [20]. In our laboratory, we have shown that synthetic hepcidin peptides inhibit *Piscirickettsia salmonis* growth, the main bacterial pathogen of salmonids [14]. These peptides inhibited bacterial growth at low concentrations and were found to internalize in the cytoplasm of *P. salmonis*, possibly affecting the replication of the pathogen. As previously noted, these types of molecules have a wide spectrum of action, and thus, their antimicrobial properties could be extrapolated to other bacterial pathogens. This study aims to assess the antimicrobial activity of synthetic variants of hepcidin against *Vibrio anguillarum* and its protective effect in the farming of the European sea bass challenged with the bacteria. Furthermore, we will determine if these types of molecules are absorbed and distributed throughout fish tissues, which will allow to better understand their possible use in commercially important fish species.

2. Materials and methods

2.1. Peptide synthesis

Synthetic peptides were synthesized by solid phase multiple peptide system using Fmoc amino acids (Iris and Rink resin 0.65 meq/g) [24]. Moreover, a portion of peptide-resin was used for Rhodamine B coupling. Then the peptides were cleaved with TFA/TIS/EDT/H_20 (92.5/2.5/2.5/2.5) (trifluoroacetic acid/triisopropylsilane/1.2-ethandithiol/ultrapure water) and purified by RP-HPLC with a 0–70% acetonitrile-water mixture gradient over 30 min at a flow rate of 1 mL/min. The peptides were lyophilized and analyzed by MALDI-TOF mass spectrometry to confirm their molecular masses. The peptides sequences (Hep1, Hep2, Hep3 and Hep4) and molecular weight are presented in the Table 1.

2.2. Peptide oxidation and characterization

The peptides were oxidized as previously was reported [14]. Briefly, 5 mg of the crude peptide were dissolved with 50% (v/v) AcOH in H_2O and subsequently diluted into 32 mL of oxidation buffer (10% isopropyl alcohol, and 10% dimethyl sulfoxide). The pH of the peptide solution was adjusted to 5.8 with NH_4OH and subjected to air oxidation at room temperature for 18 h and, prior to Sep-pak C18 purification, the pH was acidified to 2.5. To remove the salts from the crude peptide, a column that contained Sephadex G-10 eluted the mixture. The column was equilibrated with H_2O, and the crude peptides were added to the column and eluted with H_2O. Finally, the peptides coupled with Rhodamine B were applied onto a Sep-pak C18 Vac cartridge (Waters Associates) equilibrated in acidified water (0.05% trifluoroacetic acid in UPW-Ultra Pure Water). After washing with acidified water, the peptides were eluted at a flow-rate of 1 mL/min with 5%, 20%, 40%, 60% and 80% acetonitrile (ACN). The appropriate fractions were collected and the ACN was evaporated on a SpeedVac Centrifugal Evaporator. The fractions were analyzed by MALDI-TOF mass spectrometry.

2.3. Antimicrobial activity

Antibacterial activity was determined using the microplate assay as previously described [25–27]. Serial dilutions of synthetic peptides, beginning at 100 μM were mixed with 100 μL of an exponential phase bacterial culture of *V. anguillarum* (OD 0.3–0.4). The test was performed at a starting OD of 0.001 at 620 nm in tryptic soy broth containing 1.5% NaCl. After 24 h of incubation at 25 °C, absorbance values were measured and inhibition percentage was calculated.

2.4. Experimental fish

European sea bass were acclimated into fiberglass tanks of 500 L at Marine Science and Technology Park of the Universidad de Las Palmas de Gran Canaria (Las Palmas, Canary Islands, Spain). After five days of acclimatization the fish were separated into five tanks as described below. All tanks were supplied with continuously running seawater, constant aeration and a natural photoperiod (around 12 h:12 h L:D). Fish were fed daily with a commercial diet of Skretting (Burgos, Spain).

2.5. Peptide inoculation and bacterial challenge

After five days of acclimatization, 30 fish were inoculated intraperitoneally with 20 μg of Hep1 and another 30 fish were injected phosphate buffered saline. Two hours post-injection, 15 fish from each study group received a suspension of *V. anguillarum* at lethal concentration 50 (LC50) (1×10^5 CFU/mL). The four tanks

Table 1
Peptide sequences used in this study.

Peptide	Sequence	Molecular weight (Da)
Hep1	LCRWCCNCCHNKGCGFCCKF	2329.60
Hep1-Rho	Rho-LCRWCCNCCHNKGCGFCCKF	2753.46
Hep2	QSHLSLCRWCCNCCHNKGCGFCCKF	2882.20
Hep3	HSSPGGCRFCCNCCPNMSGCGVCCRF	2730.00
Hep4	QSHLSLCRWCCNCCRGNKGCGFCCKF	2958.30