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Development of a sandwich ELISA for quantifying hepcidin in Rainbow trout

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ABSTRACT

One of the most widespread antimicrobial peptides (AMPs) in fish is the hepcidins, which have potent, broad-spectrum activity against viruses, bacteria, fungi, and parasites. Moreover, they play the role of central regulation of iron metabolism and their expression is over-regulated by bacterial and viral infections, inflammation and vaccination. Quantification of their expression is an important factor in understanding their function. We therefore generated two polyclonal antibodies using synthetic peptides in order to measure hepcidin expression via sandwich ELISA. The specificity of both antibodies was confirmed by identifying an absence of cross-reactivity with other peptides that have similar pI and with the detection by Western blot of only one 9.6 kDa immunoreactive band corresponding to the hepcidin prepropeptide. The sensitivity of the sandwich ELISA was in the order of 0.005 ng/μL of hepcidin, which allowed analysis of the presence of the peptide and its variation in different tissues of *Oncorhynchus mykiss*.

With the sandwich ELISA it could be seen that hepcidin expression in rainbow trout challenged with *Aeromonas salmonicida* was increased twofold over the untreated fish in head kidney samples, in correlation with the increase in the observed transcriptional level in the head kidney cells. These results provide the first evidence for quantifying the presence of active hepcidin and may be a useful indicator of disease susceptibility, providing a new, sensitive tool for rapid screening of population health.

1. Introduction

Antimicrobial peptides (AMPs) are an integral component of the innate immunity widely distributed from invertebrates to mammals. The major groups of AMPs studied in fish include pleurocidin, piscidin, defensins, natural resistance-associated macrophage protein (Nramp), NK-lysin and hepcidin [1]. Most of them are small, cationic and hydrophobic molecules that insert into biological membranes and induce lysis in a broad-spectrum of pathogens. They may also play a role in the inflammation and modulation of the immune response [2].

Hepcidin, formerly named liver-expressed antimicrobial peptide (LEAP-1 and LEAP-2), a cysteine-rich amphipathic peptide is the most widely studied AMP. It was first identified in human liver and it plays a major role in innate immunity and iron regulation [2]. To date, hepcidin homologs have been found in various teleost fish including Nile tilapia (*Oreochromis niloticus*), Atlantic cod (*Gadus morhua*), marine medaka (*Oryzias melastigma*), orange-spotted grouper (*Epinephelus coioides*), ayu (*Plecoglossus altivelis*), large yellow croaker (*Peristernia crocea*), turbot (*Scophthalmus maximus*), Japanese sea bass (*Lateolabrax japonicas*), black porgy (*Acanthopagrus schlegelii*) and salmonids (*Salmo salar* and *Oncorhynchus mykiss*) [3]. Fish hepcidin genes consist of three exons and two introns. The first exon encodes the signal peptide and a small part of the prodomain. The other two exons encode the remaining part of the prodomain and the mature peptide [3–5]. Most of the known fish hepcidin propeptides are 85–90 amino acids in length and contain 20–26 amino acids in mature peptides [1]. The prepropeptides of fish hepcidins consist of three portions: signal peptide, prodomain, and mature peptide.

The distributions of hepcidins across fish species are different [6]. In Atlantic salmon hepcidin is expressed in the liver, blood,

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muscle, gills, and skin [7]. On the other hand, hepcidin is detected in cell line RTS11 in rainbow trout [8]. This data is evidence of their widespread presence in higher teleosts, where hepcidin mRNAs have been located in several immune tissues and cells.

Moreover, hepcidin expression in different tissues can be induced dramatically by bacterial lipopolysaccharide (LPS) and infections [9,10]. When challenged with pathogenic *Streptococcus iniae*, *Aeromonas salmonicida* and *Listonella anguillarum*, the expressional level of hepcidin was significantly up-regulated [11–13]. Therefore, it appears that hepcidin may be an effective component of the host innate immune system in response to microbial invasion and infection, making it an excellent candidate to be assessed for its capacity to indicate innate immune response in salmonids.

However, there are very few validated assays for quantifying AMP protein expression. It is well known that mRNA expression does not always reflect protein levels directly [14,15]. In addition, a single RNA transcript can be translated into more than one protein and not all transcripts are translated into functional proteins [16]. The present study reports a newly developed sandwich ELISA to quantify the expression of the antimicrobial peptide hepcidin and subsequently describes its application in the detection of this important peptide in biological samples.

2. Materials and methods

2.1. Peptide design

Peptides were designed based on the available sequence of *O. mykiss* hepcidin (GenBank Accession number: ADU85830.1). To define the best antigenic epitopes, the method of Kolaskar and Tongaonkar [17] was used in the bioinformatics server of the Immunomedicine Group of the Universidad Complutense de Madrid [http://imed.med.ucm.es/Tools/antigenic.pl]. Protscale on the Expasy server [18] was used to analyze the physicochemical behavior of the antigenic sequences and identify regions of high hydrophilicity and mean flexibility. The antigenic regions along the entire molecule were located using a human homology model (pdb: 1M4F) by applying the automatic modeling mode in the workspace of the Swissmodel server [19]. ClustalW alignment [20] was performed with trout, Atlantic salmon (*S. salar*, GenBank Accession number NP_001134321.1), Nile Tilapia (*O. niloticus*, GenBank Accession number: XP_003450578.1) and Atlantic cod (*G. morhua*, GenBank Accession number ACA42769.1) in order to identify the conserved regions. The region between amino acids 63 and 77 of the trout hepcidin prepropeptide, designated for the purposes of this report as peptide A (peptide of mature peptide hepcidin), and amino acids 64 and 88, designated as peptide B (mature peptide hepcidin), were chosen as candidate peptides for subsequent chemical synthesis.

2.2. Peptide synthesis, purification and characterization

Peptides A and B were synthesized by the solid phase multiple peptide system [21] using Fmoc amino acids (Iris and Rink resin 0.65 meq/g), cleaved with TFA/TIS/H20 (95/2.5/2.5) (trifluoroacetic acid/triisopropylsilane/ultrapure water) [Novabiochem] 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 mass. Circular dichroism (CD) spectroscopy was carried out on a JASCO J-815 CD Spectrometer coupled to a Peltier JASCO CDF-426S/15 system for temperature control (Jasco Corp., Tokyo, Japan) in the far ultra-violet (UV) range (190–250 nm) using quartz cuvettes of 0.1 cm path length and 1 nm bandwidth at 0.1 nm resolution. Each spectrum was recorded as an average of four scan repetitions in continuous scanning mode with 50 nm/min scanning speed and a response time of 1 s. The solvent contribution blank was subtracted from each sample spectrum. Molar ellipticity was calculated using 0.000043 mol/L for peptide A and 0.000049 mol/L for peptide B. CD spectra of the peptides were recorded in different solvents, such as Milli-Q water, phosphate buffer saline (PBS) and trifluoroethanol (TFE, 30% v/v in water). The spectra were recorded at 15 °C.

2.3. Fish challenged

Eight rainbow trouts (100–200 g) were purchased and maintained in 1-m-diameter fiberglass tanks supplied with recirculating fresh water at 9–12 °C (Laboratory of Applied Genetics at the Pontificia Universidad Católica de Valparaíso, CHILE). Fishes were fed twice daily with a commercial pellet trout diet. The trouts were separated into two groups: a control group and those challenged with *A. salmonicida*.

The fish were anesthetized with benzocaine (25 μg/L) prior to treatment. The fish challenges were performed by intraperitoneal (i.p) injections of 1.2 mg of *A. salmonicida* and phosphate buffered saline (PBS) (control group).

All trout were sampled 12 h after challenge. The fish were killed by immersion in a solution of 50 mg/L benzocaine (BZ-20), until no signs of movement or reflexes were observed. Head kidney samples were then collected and resuspended directly in lysis buffer (50 mM Tris–HCl, pH 7.6, 0.3 M NaCl, 0.5% Triton X-100, 0.5 M EDTA and 0.2% protease inhibitor cocktail) for protein analysis.

2.4. Antibody production

Polyclonal antibodies were generated against peptide A in six week-old female CF-1 mice. These mice were administered intraperitoneal injections at 0, 7, 14 and 21 days with 60 μg of peptide each, diluted 1:1 in FIS (FISEAIIHVLHSR) as a T helper cell activator [22], and 500 μL of Freund's adjuvant (Thermo). At day 30, the mice were anesthetized with a small dose of chloroform and bled with a hypodermic needle 18G*1 ½″ (NIPRO). 2 mL of antiserum was collected from each mouse and centrifuged at 1000 g for 5 min. The serum was then stored at −20 °C.

In addition, polyclonal antibodies were generated against peptide B in New Zealand rabbits, which were administered subcutaneous injections at 0, 15 and 30 days with 500 μg immunogen diluted 1:1 in FIS, and 500 μL of Freund's adjuvant (Thermo). 20 mL of antiserum were collected on day 45 and then centrifuged at 1000 g for 5 min. The serum was then stored at −20 °C. In both cases, preimmune control serum was obtained prior to immunization.

2.5. Antibody characterization

The specificity of the antiserums (mouse immune sera anti-peptide A and rabbit immune sera anti-peptide B) was evaluated by immunoblotting and indirect ELISA using peptides A and B and other peptides with similar pI as antigens. For the case of immunoblotting, the peptides were placed onto a nitrocellulose membrane (Thermo 0.45 μm) seeding 4, 2, 1, 0.5, 0.25 and 0.125 μg of each. The membrane was blocked with 3% Bovine serum albumin (BSA) in PBS for 60 min at 20 °C, and washed twice with PBS and once with PBS containing 0.05% Tween-20 (PBST). Thereafter, the membrane was incubated with a 1:2000 dilution of sera overnight at 20 °C. After three washings with PBST, anti-IgG mouse-HRP (Thermo) diluted 1:7000 or anti-IgG rabbit-HRP (Thermo) diluted 1:5000 was added and this was then incubated for 1 h at 20 °C.