Fish & Shellfish Immunology 28 (2010) 235–239

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Short communication

An immunological method for quantifying antibacterial activity in *Salmo salar* (Linnaeus, 1758) skin mucus

Edgar Narvaez [a], Jorge Berendsen [a], Fanny Guzmán [a,c], José A. Gallardo [b], Luis Mercado [a,c,*]

[a] Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile
[b] Laboratorio de Genética Aplicada, Escuela de Ciencias del Mar, Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile
[c] Núcleo Biotecnológico de Curauma (NBC), Pontificia Universidad Católica de Valparaíso, Valparaíso, Chile

ARTICLE INFO

Article history:
Received 3 July 2009
Received in revised form
25 September 2009
Accepted 4 October 2009
Available online 14 October 2009

Keywords:
Fish antibacterial peptide
Antibacterial quantification
Salmo salar
SAMP H1

ABSTRACT

Antimicrobial peptides (AMPs) are a pivotal component of innate immunity in lower vertebrates. The aim of this study was to develop an immunological method for quantifying AMPs in *Salmo salar* skin mucus. A known antimicrobial peptide derived from histone H1 previously purified and described from *S. salar* skin mucus (SAMP H1) was chemically synthesized and used to obtain antibodies for the quantification of the molecule via ELISA. Using skin mucus samples, a correlation of bacterial growth inhibition versus SAMP H1 concentration (ELISA) was established. The results provide the first evidence for quantifying the presence of active AMPs in the skin mucus of *S. salar* through the use of an immunological method.

1. Introduction

Fish live in a microbe-rich environment and are vulnerable to invasion by pathogenic or opportunistic microorganisms [1,2]. Teleost fish rely heavily upon innate or non-specific immune mechanisms for initial protection against these infectious agents [3,4]. The epithelial surfaces of fish, such as the skin, gills and the alimentary tract, provide first contact with potential pathogens [5,6]. These surfaces are covered by a layer of mucus composed of biochemically diverse secretions from epidermal goblet cells, which contain a number of molecular compounds such as complement, transferrin, lysozyme, C-reactive protein and antimicrobial proteins and peptides [4]. Antimicrobial peptides (AMPs) play an important role in the innate immunity of lower vertebrates [5,7,8], they demonstrate potent, broad–spectrum activity against viruses, bacteria, fungi and parasites [9]. Although these AMPs are heterogeneous with respect to their primary structure, they are mostly positively charged, amphipathic, and can form α-helical or β-sheet structures in membrane-like environments, leading to membrane destabilization and channel formation in bacterial cells [10,11]. The expression of AMPs in most fish is constitutive in different tissues such as skin, gill, kidney and gut [12–15], therefore the amount of active peptides could be a fine indicator of robust immunity [16–19]. Several antimicrobial peptides described from fish have been found to be molecules derived from proteins that are not traditionally associated with antimicrobial defense, such as histones, which are small basic proteins most commonly found in association with DNA in the chromatin [20,21]. The following peptides derived from histones have been identified as antimicrobial: Parasin I from catfish [12] Oncorhyncin II and III from rainbow trout [22,23], Hipossin from Atlantic halibut [24] and SAMP H1 derived from Histone H1 from Atlantic salmon [25].

Considering that antimicrobial peptides are expressed in the skin mucus of fish constitutively for protection against pathogens, we propose that measuring antibacterial activity could be an appropriate and simple indicator of robust innate immunity. Normally this is evaluated using a bacterial growth inhibition test [26,27]. The aim of this work was to establish a correlation between antibacterial activity and immunological quantification of SAMP H1 from *Salmo salar* skin mucus. The expression of this AMP, quantified by ELISA, may be a useful indicator of disease susceptibility, providing a new and sensitive tool for rapid screening, at low cost, of innate immunity level in teleost fish.

2. Material and methods

In all experimental procedures we utilized farmed Atlantic salmon (*S. salar*) with weights ranging from 200 to 300 g and a mean size of

* Corresponding author at: Laboratorio de Genética e Inmunología Molecular, Instituto de Biología, Pontificia Universidad Católica de Valparaíso, PO Box 4059, Valparaíso, Chile. Tel./fax: +56 032 227 31 25.
E-mail address: lmercado@ucv.cl (L. Mercado).

1050-4648/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.fsi.2009.10.004

19 cm that were maintained and stabilized in laboratory culture conditions. The fish were humanely killed by immersion in a solution of 50 mg/L benzocaine (BZ-20) until no signs of movement or reflexes were observed. Skin mucus was collected with a Cell Scraper (Orange Scientific) from the entire dorso-lateral area of the fish, avoiding sample contamination with blood, scales or intestinal fluids. Ten individual skin mucus samples were homogenized 1:4 (v/v) in Buffer A (BA) 50% (v/v) ethanol (Merck), 3.3% trifluoroacetic acid (TFA) (Merck) and 2% general use protease inhibitor cocktail (Sigma). The samples were mixed for 5 min in a vortex 59 (Denville Scientific Inc.) and sonicated for 3 × 30 s at 11 RMS in ice. The resulting samples were shaken at 100 RPM overnight at 4 °C. The preparation was centrifuged at 11,000 × *g* for 45 min at 4 °C and the supernatant rich in AMPs was stored at −80 °C until use.

Antibacterial activity of each skin mucus sample from *S. salar* was determined by the microplate assay described by Mercado et al. [26]. *Vibrio ordalii* (ATCC 33509) was incubated in Tryptic Soy Broth (TSB) at 37 °C until reaching an optical density (OD) of 0.2–0.3 at 620 nm. Antibacterial activity was determined by incubating standard aliquots of 100 μl of a 1:100 dilution of the bacterial suspension in duplicate, exposed to 10 μl of skin mucus sample. After 24 h of incubation at 37 °C, absorbance values at 620 nm were determined. Percentage growth inhibition was determined by subtracting the growth values of the bacteria exposed to different concentrations of the target molecules from the bacterial growth values in the absence of peptides. As a positive control for growth inhibition, synthetic peptide was used at a concentration of 1 μM; an amount known to fully inhibit Gram negative bacterial growth [27].

The antigenic properties of SAMP H1 were analyzed using bioinformatic tools based on the primary sequence: AEVAPAPAAAA PAKAPKKKAAAKPKKAGPS [25]. CLC Main Workbench 5.0 (CLC bio USA, Cambridge, MA, USA) was used to recognize hydrophilic and flexible regions, as indicators of the presence of epitopes.

The known sequence of SAMP H1 was synthesized using standard Fmoc chemistry as described by Houghten [28]. The peptides were cleaved by TFA/TIS/H_20 (95/2.5/2.5) and purified by RP-HPLC to a purity of >95%. The molecular mass of the purified peptide was confirmed by mass spectrometry MALDI-TOF [27].

CF-1 Mice were obtained from the Public Health Institute, Chile. Immunization took place at days 0, 7, 14 and 21 by intraperitoneal injection with 60 μg of the synthetic peptide, FIS (1:1), as a T helper cell activator [29] and 500 μl of Complete Freund's adjuvant (Thermo). On day 6, 0.6 ml of Pristane (2,6,10,14-tetramethylpentadecane) (Sigma) was injected to induce tumor formation [30]. On day 30, mice were anesthetized with small doses of chloroform and their ascitic fluids were drained, centrifuged at 300 × *g* for 10 min and the supernatant rich in mouse IgG was recovered, then immunoaffinity chromatography using Cyanogen Bromide-Activated Sepharose 4B (Sigma–Aldrich) was performed as follows [31]. Mouse ascitic fluids were filtered through a 0.22 μm filter (Millipore) and diluted one to two times with phosphate buffered saline (PBS, pH 7.4). Then 0.5 g of gel was prepared with 2 ml of 1 mM HCl. The synthetic SAMP H1 (1 mg) was then coupled with the prepared gel in coupling buffer (0.1 M NaHCO3, pH 8.3, containing 0.5 M NaCl). The unreacted active groups were blocked with 2 M glycine. Finally, the prepared mouse ascitic fluids were mixed with the sepharose-coupled peptide. The mixture was rotated overnight at 4 °C and subsequently the gel was washed with PBS (pH 7.4). It was then eluted with 1 mM HCl (pH 3.0) and the monospecific antibodies were collected in 0.05 mM Tris-base (pH 9.5) and stored at −20 °C.

The affinity-purified antibody against SAMP H1 was tested by dot blot assay, performed in nitrocellulose membrane 0.45 μm (Thermo) seeding 2 μg, 1 μg of the synthetic peptide and a control dot with 0.5 μl of mouse IgG. A western blotting assay was used to confirm the dose-response of SAMP H1 in skin mucus samples. Serial dilutions (5, 10, 15 and 20 μg) were electrotransfered to PVDF membrane and were blocked using 3% BSA (Boval) in PBS for 1.5 h at 37 °C. After blocking, the membrane was incubated in 3 μg/ml of primary antibody (affinity-purified antibody) for 1.5 h at 37 °C, followed by incubation in a secondary antibody (goat IgG anti-IgG mouse-HRP (Sigma) (1:7000)) at 37 °C for 1 h. The peptide was observed with 3,3 diaminobenzidine (Aldrich) in the presence of hydrogen peroxide.

The ELISA standard curve for quantifying SAMP H1 used two-fold serial dilutions ranging from 10.0 to 0.156 ng/μl of pure synthetic SAMP H1 in 96-well microtiter plates. 2 μg of skin mucus samples described above were coated overnight at room temperature with 100 μl/well $NaHCO_3$ (0.06 M, pH 9.6) buffer. To validate the assay a recovery test was performed. Samples of the ten fish were spiked with synthetic SAMP H1 at a final concentration of 1.5 and 3.0 ng/μl. SAMP H1 concentration was measured in duplicate a total of two times and percent recovery was calculated according to Corrales et al. [40].

To correlate ELISA quantification of SAMP H1 with its antibacterial activity, sample concentration used for each assay were the same. Microplates were washed 3 times with PBS-Tween 0.05% (200 μl/well) between every step of the assay and non-specific sites were blocked for 4 h at 37 °C using 5% BSA in PBS (200 μl/well). Mouse IgG anti-SAMP H1 was used at 3 μg/ml in PBS incubated for 90 min at 37 °C, secondary antibody anti-mouse IgG-HRP (Sigma) diluted 1:20,000 in PBS was incubated for 60 min at 37 °C and subsequently TMB peroxidase substrate (Invitrogen) was added and incubated for 30 min at room temperature. The reaction was stopped with 50 μl of 2 N sulfuric acid and the absorbance was measured at 450 nm with an ELISA reader (Versamax). Finally, comparison between the fish with the highest and lowest antibacterial activity was performed to establish a linear correlation between antibacterial activity and SAMP H1 concentration via optical density.

For statistical analysis, calculations of means, standard deviations and analysis of variance (ANOVA) of skin mucus samples were carried out using Microsoft Excel 2007 and GraphPad Prism software 5.0 (GraphPad software, San Diego, CA). Differences were considered significant if the *p*-value was <0.05.

3. Results and discussion

Fish are exposed to a pathogen-rich environment, depending on their skin mucus as primary protection against potential pathogens. Within this layer are a variety of antibacterial components such as AMPs, lysozyme, proteases and lectins [1,32,33]. The aim of this research was to standardize an immunological method for quantifying antibacterial activity in skin mucus of *S. salar*. The first step was to obtain a monospecific antibody against SAMP H1, and thus, a bioinformatic analysis was performed using CLC Main Workbench [34,35]. The primary structure analysis of SAMP H1 indicates at least two probable epitope regions based on low hydrophobicity and chain flexibility properties (Fig. 1A). The most probable conformation is a loop structure, as this region is more exposed and would thus become a zone of antibody recognition (Fig. 1B) [36,37]. Considering the bioinformatic analysis of SAMP H1 sequence, we decided to use it for immunization. Chemical synthesis is one of the primary strategies for fast generation of epitope sequences which are utilized for obtaining antibodies. The peptide was synthesized using Fmoc strategy and its purity and molecular mass confirmed by RP-HPLC and MALDI-TOF MS (data not shown). Immunorecognition of SAMP H1 was demonstrated via dot blot, using the IgGs generated in CF-1 mouse ascites tumors as antibodies (Fig. 1C). Antibody recognition for the antigen was confirmed by ELISA, whose linear regression ($r^2 = 0.98$) is sufficient proof (Fig. 1C). To demonstrate the utility of this antibody on biological samples, western blotting analysis of skin

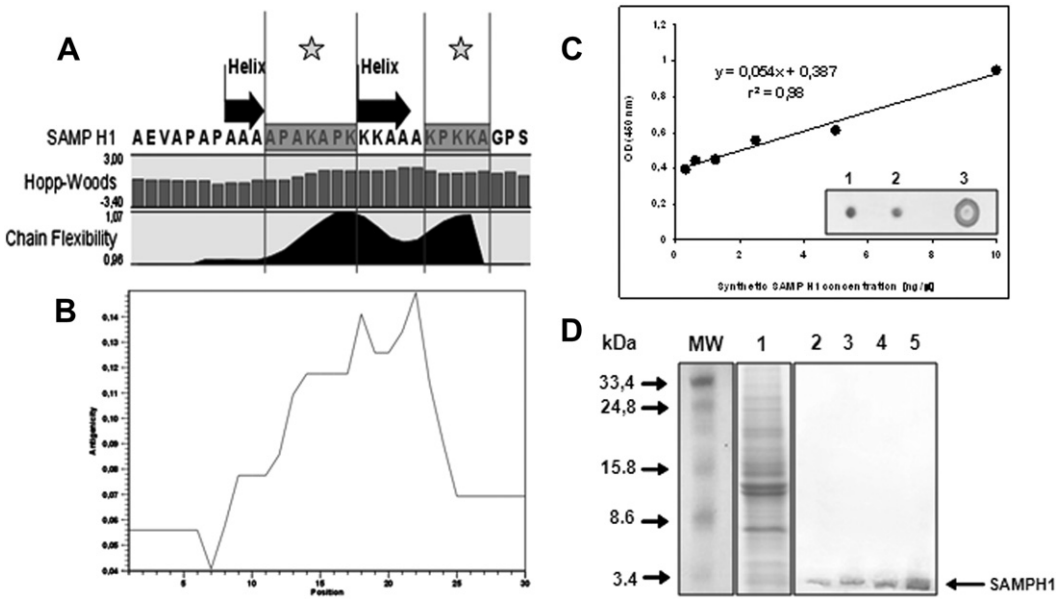

Fig. 1. (A) Epitope regions in SAMP H1 sequence. Symbols show probable regions that are most likely antigenic (★). Hydrophobicity and chain flexibility are also shown. (B) Antigenicity plot showing residues that are antigenic. (C) Quantitative and qualitative immunorecognition of SAMP H1. Linearity of the synthetic SAMP H1 dilution curve, two-fold serial dilution of (10.0–0.156 ng/μl) was used. The curve corresponds to an average of 4 experiments. Insert shows positive Dot blotting assay. (D) 18% SDS/PAGE Tris/Tricine (lane 1) and western blotting serial dilutions (lane 2 to 4) of skin mucus sample.

mucus confirmed the presence of the molecule in this tissue, corroborating its major expression and localization that were previously proposed [25]. Moreover, the increase in the detection of SAMP H1 with the use of an increased concentration of the skin mucus sample, confirms the specificity of the antibody (Fig. 1D). In order to back up this result, a recovery efficiency ELISA was established by adding synthetic SAMP H1 to skin mucus samples. Concentration of endogenous SAMP H1 samples and expected recovery, observed recovery and percentage recovery of ten fish samples spiked with synthetic SAMP H1 are shown in Table 1. From the twenty tested samples an optimal recovery percentage> 80% was obtained. The average of the recovery percentage with the addition of 1.5 and 3.0 ng/μl of the synthetic peptide is slightly favourable when 1.5 ng/μl are added. These results indicate the utility of the antibody for specific SAMP H1 recognition in skin mucus samples, which is analogous to the validation carried out for other antibacterial peptides [40] (Fig. 2).

Once the utility of the antibody in detecting SAMP H1 had been confirmed, attention was centered on standardizing a method for the quantification of its antibacterial activity using a growth inhibition microplate assay. The first step was to establish the optimal biochemical extraction condition. Unlike the protocol used for the extraction of SAMP H1 by Lüders et al. [25], another method was chosen using BA, which has been used to characterize another AMP, Oncorhyncin III [2], and has shown more efficacy in SAMP H1 extraction. Despite Lüders use of an acidic compound (acetic acid 2%), TFA is a much stronger acid and the use of protease inhibitor cocktail prevented the degradation of the molecules present in the solution, therefore better AMP extraction was obtained. This was demonstrated using antibacterial activity test that showed that BA was in fact the better solution for obtaining active AMPs (data not shown). Additionally SAMP H1 concentration values detected in the skin mucus of *S. salar* are between 2.0 and 3.0 μM, which are similar to those obtained by other authors [2,25].

Given that antimicrobial peptides are constitutive in different tissues such as skin, liver, gut, among others [12–15], and that SAMP H1 is the dominant antimicrobial peptide in *S. salar* skin mucus preparations [25], the level of differential expression between specimens is a property that has not been used as an innate immunity parameter, so when SAMP H1 was purified and characterized this was not established [25].

Table 1
Recovery of SAMP H1 by ELISA from *S. salar* skin mucus samples supplemented with synthetic SAMP H1.

Fish	Endogenous SAMP H1 (ng/ul) (mean ± SD)	Amount of synthetic SAMP H1 added					
		1.5 ng/μl			3.0 ng/μl		
		Expected	Observed	% Recovery	Expected	Observed	% Recovery
1	0.31 ± 0.02	1.81	1.77 ± 0.01	**98**	3.31	2.73 ± 0.03	**82**
2	0.08 ± 0.03	1.58	1.77 ± 0.03	**112**	3.08	3.20 ± 0.19	**104**
3	0.24 ± 0.18	1.74	1.82 ± 0.07	**105**	3.24	3.42 ± 0.03	**106**
4	0.58 ± 0.01	2.08	1.96 ± 0.03	**94**	3.58	3.78 ± 0.01	**106**
5	1.00 ± 0.05	2.50	2.48 ± 0.03	**99**	4.00	4.19 ± 0.06	**105**
6	0.55 ± 0.04	2.05	1.70 ± 0.03	**83**	3.55	4.41 ± 0.04	**124**
7	0.74 ± 0.01	2.24	1.97 ± 0.05	**88**	3.74	4.45 ± 0.01	**119**
8	0.31 ± 0.04	1.81	2.31 ± 0.03	**127**	3.31	3.45 ± 0.04	**103**
9	1.07 ± 0.06	2.57	3.05 ± 0.15	**119**	4.07	3.62 ± 0.06	**89**
10	0.73 ± 0.06	2.23	2.33 ± 0.02	**104**	3.73	4.31 ± 0.06	**116**
Mean				**103**			**105**

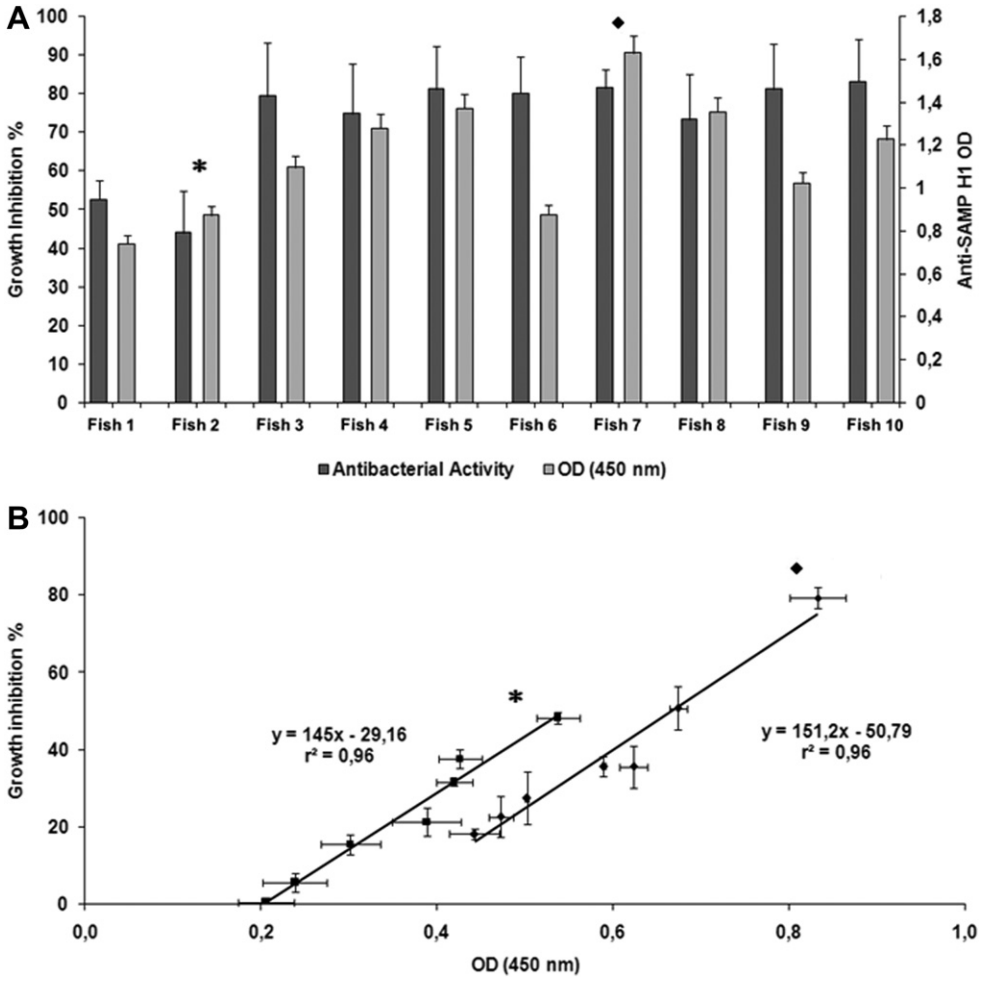

Fig. 2. (A) Comparison between antibacterial activity and SAMP H1 quantification of *Salmo salar* skin mucus. Dark column: antibacterial activity, light column: ELISA SAMP H1 quantification (OD 450 nm). (B) Linear correlation of individual skin mucus between antibacterial activities and concentration of SAMP H1 from fish with low antibacterial activity (∗: Fish 2) and high antibacterial activity (◆: Fish 7).

Our results show that skin mucus antibacterial activity was different between specimens (Fig. 2A dark columns), this suggests that individual differences do exist, this could be due to different levels of SAMP H1 expression, thus becoming a possible parameter of robust immunity of fish. It should be considered that sampling conditions may influence the quantification of the parameter [38], which in our experiments has been protected against through standardizing the fish culture and the management of sample-taking. When the level of antibacterial activity of the skin mucus samples of each fish was analyzed, the presence of SAMP H1 was also quantified, and a proportional trend was observed between both parameters (Fig. 2A, light column). Therefore the choice of two samples, fish 2 (low expression and low antibacterial activity) and fish 7 (high expression and high antibacterial activity) enabled mathematical correlation (high linear regression) of the fact that the SAMP H1 level detected by ELISA reflects the presence of the active molecule in the skin mucus (Fig. 2B).

On the other hand robust and economical procedures to quantify immunological genetic variation are widely required in fish breeding to improve the health and welfare of fish in farm conditions. The quantification of skin mucus antibacterial activity using ELISA as described here could be used as a fast and cheap indicator of innate immunity to improve disease resistance of various fish species [39]. This research describes the first step in generating a method for screening robust immunity in farmed salmon. The next step will be to correlate high SAMP H1 expression with fish that survive pathogen challenge. Recently, an ELISA for the quantification of piscidin 4 was developed, which at the time of our investigation was published by the group of E.J. Noga [40]. They have demonstrated that healthy fish have more AMP expression than feed-restricted and diseased (*Ichthyobodo necator* ectoparasite infestation) fish, although their biological model and studied AMPs were different from those of this research. The proposal of using skin mucus is most advantageous because it could allow sampling without sacrificing the animal.

Acknowledgements

This work was supported by Grant 07CN13PBT-61 of InnovaCHILE-CORFO and AQUAINNOVO and INTESAL companies. We thank the staff of AQUACHILE for their assistance in the collection of fish and the members of the Laboratorio de Genética Aplicada (PUCV) for the fish care during this research. We thank Mr. James Conaghan for his English review assistance.

References

[1] Ellis AE. Innate host defense mechanisms of fish against viruses and bacteria. Developmental and Comparative Immunology 2001;25:827–39.

[2] Fernandes JMO, Nathalie S, Kemp GD, Smith VJ. Oncorhyncin III: a potent antimicrobial peptide derived from the non-histone chromosomal protein H6 of rainbow trout, *Oncorhynchus mykiss*. Biochemical Journal 2003;373:621–8.
[3] Nagashima Y, Kikuchi N, Shimakura K, Shiomi K. Purification and characterization of an antibacterial protein in the skin secretion of rockfish *Sebastes schlegeli*. Comparative Biochemistry and Physiology Part C 2003;136:63–71.
[4] Subramanian S, MacKinnon S, Ross N. A comparative study on innate immune parameters in the epidermal mucus of various fish species. Comparative Biochemistry and Physiology Part B 2007;148:256–63.
[5] Robinette D, Wada S, Arroll T, Levy MG, Miller WL, Noga EJ. Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like antimicrobial proteins. Cellular and Molecular Life Sciences 1998;54:467–75.
[6] Iijima N, Tanimoto N, Emoto Y, Morita Y, Uematzu K, Murakami T, et al. Purification and characterization of three isoforms of chrysophsin, a novel antimicrobial peptide in the gills of the red sea bream, *Chrysophrys major*. European Journal of Biochemistry 2003;270:675–86.
[7] Cho S-H, Lee B-D, An H, Eun J-B, Kenojeinin I. Antimicrobial peptide isolated from the skin of the fermented skate, *Raja kenojei*. Peptides 2005;26:581–7.
[8] Noga EJ, Silphaduang U, Park NG, Seo JK, Stephenson J, Kozlowicz S. Piscidin 4: a novel member of the piscidin family of antimicrobial peptides. Comparative Biochemistry and Physiology Part B 2009;152:299–305.
[9] Marshall SH, Arenas G. Antimicrobial peptides: a natural alternative to chemical antibiotics and a potential for applied biotechnology. Electronic Journal of Biotechnology 2003;6:271–84.
[10] Jenssen H, Hamill P, Hancock RE. Peptide antimicrobial agents. Clinical Microbiology Reviews 2006;19:491–511.
[11] Kitani Y, Tsukamoto C, Zhang G, Nagai H, Ishida M, Ishizaki S, et al. Identification of an antibacterial protein as L-amino acid oxidase in the skin mucus of rockfish *Sebastes schlegeli*. The FEBS Journal 2006;274:125–36.
[12] Park IY, Park CB, Kim MS, Kim SC. Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*. FEBS Letters 1998;437:258–62.
[13] Lauth X, Shike H, Burns JC, Westerman ME, Ostland VE, Carlberg JM, et al. Discovery and characterization of two isoforms of moronecidin, a novel antimicrobial peptide from hybrid striped bass. Journal of Biological Chemistry 2002;277:5030–9.
[14] Zhang YA, Jun Zou, Chang C, Secombes CJ. Discovery and characterization of two types of liver-expressed antimicrobial peptide 2 (LEAP-2) genes in rainbow trout. Veterinary Immunology and Immunopathology 2004;101:259–69.
[15] Zou J, Mercier C, Koussounadis A, Secombes C. Discovery of multiple beta-defensin like homologues in teleost fish. Molecular Immunology 2007;44:638–47.
[16] Bayne C, Gerwick L. The acute phase response and innate immunity of fish. Developmental and Comparative Immunology 2001;25:725–43.
[17] Gerwick L, Corley-Smith G, Bayne CJ. Gene transcript changes in individual rainbow trout livers following an inflammatory stimulus. Fish & Shellfish Immunology 2007;22:157–71.
[18] Alvarez-Pellitero P. Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. Veterinary Immunology and Immunopathology 2008;126:171–98.
[19] Sullivan C, Kim CH. Zebrafish as a model for infectious disease and immune function. Fish & Shellfish Immunology 2008;25:341–50.
[20] Bergsson G, Agerberth B, Jörnvall H, Gudmundsson GH. Isolation and identification of antimicrobial components from the epidermal mucus of Atlantic cod (*Gadus morhua*). FEBS Journal 2005;272:4960–9.
[21] Parseghian MH, Luhrs KA. Beyond the walls of the nucleus: the role of histones in cellular signaling and innate immunity. Biochemistry and Cell Biology 2006;84:589–604.
[22] Fernandes JMO, Kemp GD, Molle MG, Smith VJ. Anti-microbial properties of histone H2A from skin secretions of rainbow trout, *Oncorhynchus mykiss*. Biochemical Journal 2002;368:611–20.
[23] Fernandes JMO, Molle MG, Kemp GD, Smith VJ. Isolation and characterization of oncorhyncin II, a histone H1-derived antimicrobial peptide from skin secretions of rainbow trout, *Oncorhynchus mykiss*. Developmental and Comparative Immunology 2004;28:127–38.
[24] Birkemo GA, Lüders T, Andersen ∅, Nes IF, Nissen-Meyer J. Hipposin, a histone-derived antimicrobial peptide in Atlantic halibut (*Hippoglossus hippoglossus* L.). Biochimica Biophysica Acta 2003;1646:207–15.
[25] Lüders T, Birkemo GA, Nissen-Meyer J, Andersen ∅, Nes IF. Proline conformation-dependent antimicrobial activity of a proline rich histone H1 N-terminal peptide fragment isolated from the skin mucus of Atlantic salmon. Antimicrobial Agents and Chemotherapy 2005;49:2399–406.
[26] Mercado L, Schmitt P, Marshall SH, Arenas G. Gill tissues of the mussel *Mytilus edulis chilensis*: a new source for antimicrobial peptides. Electronic Journal of Biotechnology 2005;8:284–90.
[27] Schmitt P, Mercado L, Dìaz M, Guzmán F, Arenas G, Marshall SH. Characterization and functional recovery of a novel antimicrobial peptide (CECdir-CECret) from inclusion bodies after expression in *Escherichia coli*. Peptides 2008;29:512–9.
[28] Houghten R. General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids. Proceedings of the National Academy of Sciences 1985;82:5131–5.
[29] Prieto I, Herras-Stubbs S, Garcia-Granero M, Berasain C, Riezu-Boj J-I, Lasarte J-J, et al. Simple strategy to induce antibodies of distinct specificity: application to the mapping of gp120 and inhibition of HIV-1 infectivity. European Journal of Immunology 1995;25:877–83.
[30] Mercado L, Cao A, Barcia R, Ramos-Martùnez JI. Purification of a lipid-activated and Ca^{2+}-independent protein kinase from the mantle tissue of *Mytilus galloprovincialis* Lmk. Molecular and Cellular Biochemistry 2002;233:99–105.
[31] Jin J-F, Yuan L-D, Liu L, Zhao Z-J, Xie W. Preparation and characterization of polyclonal antibodies against ARL-1 protein. World Journal of Gastroenterology 2003;9:1455–9.
[32] Fast MD, Sims DE, Burka JF, Mustafa A, Ross NW. Skin morphology and humoral non-specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. Comparative Biochemistry and Physiology Part A Molecular and Integrative Physiology 2002;132:645–57.
[33] Subramanian S, Ross NW, MacKinnon SL. Myxinidin, a novel antimicrobial peptide from the epidermal mucus of Hagfish, *Myxine glutinosa* L. Marine Biotechnology, in press doi: 10.1007/s10126-009-9189-y.
[34] Moreau V, Fleury C, Piquer D, Nguyen C, Novali N, Villard S, et al. PEPOP: computational design of immunogenic peptides. BMC Bioinformatics 2008;9:71.
[35] Odorico M, Pellequer J-L. BEPITOPE: predicting the location of continuous epitopes and patterns in proteins. Journal of Molecular Recognition 2003;16:20–2.
[36] Hoop TP, Woods KR. Prediction of protein antigenic determinants from amino acid sequences. Proceedings of the National Academy of Sciences USA 1985;78:3824–8.
[37] Tainer JA, Getzoff ED, Alexander H, Houghten RA, Olson AJ, Lerner RA, et al. The reactivity of anti-peptide antibodies is a function of the atomic mobility of sites in a protein. Nature 1984;312:127–34.
[38] Miranda CD, Kehrenberg C, Ulep C, Schwarz S, Roberts MC. Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. Antimicrobial Agents and Chemotherapy 2003;47:883–8.
[39] Sitjà-Bobadilla A. Living of a fish: a trade-off between parasites and the immune system. Fish & Shellfish Immunology 2008;25:358–72.
[40] Corrales J, Gordon WL, Noga EJ. Development of an ELISA for quantification of the antimicrobial peptide piscidin 4 and its application to assess stress in fish. Fish & Shellfish Immunology 2009;27:154–63.