ABSTRACT

ULLAL, ANIRUDH JAIWANT. Extraerythrocytic expression of the β-subunit of hemoglobin is associated with a potent anti-parasitic defense in fish. (Under the direction of Dr. Edward J. Noga.)

Innate immunity plays a crucial role in the defense against prokaryotic and eukaryotic pathogens. Major components of this defense are antimicrobial peptides (AMPs). Some AMPs are derived from larger proteins with other recognized functions (e.g., lactoferrin, histones). In this study, we demonstrate the expression of peptides homologous to the β-chain of hemoglobin (Hb-β), one of the two major subunits of this respiratory protein. These Hb-β peptides (HbβP-1, -2 and -3), isolated from gill of the economically important channel catfish (*Ictalurus punctatus*), had antibacterial activity and were upregulated in gill and skin epithelium in response to parasitic (*Ichthyophthirius multifiliis*, ich) infection. One peptide (HbβP-1), while having relatively weak antibacterial activity had antiparasitic activity comparable to that of other potently antiparasitic AMPs. Also, this cidal activity was specifically directed against the trophozoite (trophont) stage of ich at a low concentration (6.2 μg/ml, 1.7 μM) but had no apparent effect on the disseminative (theront) stage or the reproductive (tomont) stage at the highest concentration tested (400 μg/ml, 108 μM). In addition, HbβP-1 was not lytic to channel catfish erythrocytes at the highest concentration tested (400 μg/ml, 108 μM). Immunohistochemistry and *in situ* hybridization of skin and gill from fish experimentally challenged with ich indicated that the HbβP-1 sequence was both synthesized and expressed in epithelial tissues of skin and gill, which are the target tissues for ich. “Bug blots” of gill extracts from fish recovering from a bacterial infection suggested that upregulation of these Hb-β related peptides might also occur with other infections. These findings, along with the recent discovery by others that Hb-β is expressed in mammalian

macrophages and alveolar epithelium, suggest that hemoglobin-derived AMPs might play a significant role in the non-specific immune response of vertebrates.

EXTRAERYTHROCYTIC EXPRESSION OF THE β-SUBUNIT OF HEMOGLOBIN IS ASSOCIATED WITH A POTENT ANTI-PARASITIC DEFENSE IN FISH

by
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DEDICATION

To My Family - my eternal source of love, support and wisdom

BIOGRAPHY

Anirudh Jaiwant Ullal was born to Dr. Mrs. Bharati J. Ullal and Mr. Jaiwant G. Ullal on the twenty-ninth day of September, in the year 1977 and immediately set about subjecting his most wonderful parents to sleepless nights. Born and raised in Mumbai, India, he was always encouraged to mature as an independent thinker and quickly learned from example that patience is truly a virtue. The elder of two brothers, and amongst the youngest of numerous cousins, he enjoyed a fulfilling childhood in a close-knit family. He was graced by the immeasurable love and wisdom of doting grandparents and granduncles and the unfaltering affections of all his aunts and uncles.

His educational path took him from kindergarten through eighth grade at Hasanat High School, before graduating high school from Rishi Valley School. He was fortunate for the tutelage of educators par excellence throughout. He completed two years of junior college at Ramnaraian Ruia College, Mumbai and continued on to pursue a bachelors degree in Life Science, graduating in 1998. During completion of a Masters program in Biotechnology at the University of Mumbai, India in 2000, he was accepted into the doctoral program in Comparative Biomedical Science at North Carolina State University's College of Veterinary Medicine. In 2006, he received his Ph.D. following six years of stimulating research in innate host defense mechanisms under the able guidance of Dr. Edward J. Noga. He continues to seek new challenges in scientific research.

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Chapter 1

Extra-erythrocytic expression of antimicrobial peptides derived from the β-subunit of hemoglobin is associated with a potent anti-parasitic defense in fish

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ABSTRACT

Innate immunity plays a crucial role in the defense against prokaryotic and eukaryotic pathogens. Major components of this defense are antimicrobial peptides (AMPs). Some AMPs are derived from larger proteins with other recognized functions (e.g., lactoferrin, histones). In this study, we demonstrate the expression of peptides homologous to the β-chain of hemoglobin (Hb-β), one of the two major subunits of this respiratory protein. These Hb-β peptides (HbβP-1, -2 and -3), isolated from gill of the economically important channel catfish (*Ictalurus punctatus*), had antibacterial activity and were upregulated in gill and skin epithelium in response to parasitic (*Ichthyophthirius multifiliis*, ich) infection. One peptide (HbβP-1), while having relatively weak antibacterial activity, had antiparasitic activity comparable to that of other potently antiparasitic AMPs. Also, this cidal activity was specifically directed against the trophozoite (trophont) stage of ich at a low concentration (6.2 μg/ml, 1.7 μM) but had no apparent effect on the disseminative (theront) stage or the reproductive (tomont) stage at the highest concentration tested (400 μg/ml, 108 μM). In addition, HbβP-1 was not lytic to channel catfish erythrocytes at the highest concentration tested (400 μg/ml, 108 μM). Immunohistochemistry and *in situ* hybridization of skin and gill from fish experimentally challenged with ich indicated that the HbβP-1 sequence was both synthesized and expressed in epithelial tissues of skin and gill, which are the target tissues for ich. "Bug blots" of gill extracts from fish recovering from a bacterial infection suggested that upregulation of these Hb-β related peptides might also occur with other infections. These findings, along with the recent discovery by others that Hb-β is expressed in mammalian macrophages and alveolar epithelium, suggest that hemoglobin-derived AMPs are evolutionarily conserved and might play a significant role in the non-specific immune response of vertebrates.

INTRODUCTION

The innate immune system is the sole defense mechanism of invertebrates and a fundamental defense in lower vertebrates, such as fish. It is able to recognize conserved patterns in molecular structures that are characteristically non-self, shared by a large group of pathogens called pathogen-associated molecular patterns (PAMPs) through nonclonal receptors of broad specificity called pattern recognition receptors (PRRs). This recognition induces an immediate cellular as well as non-cellular response resulting in phagocyte recruitment, complement activation as well as production of cytokines. On the other hand, the adaptive immune system in higher vertebrates consists of somatically produced T and B cell surface receptors, which are less discriminating between self and non-self and hence requires a secondary signal to confirm pathogen recognition, usually provided by the previously activated innate immune system (Medzhitov and Janeway 1997). Thus, the innate immune system serves an instructive role in the acquired immune response and hence plays an equally important role in higher vertebrates (Magnadóttir 2006; Fearon 1996, 1997). The innate immune system includes physical barriers like skin and mucus, along with non-specific components of the cellular and non-cellular immune response systems (Magnadóttir 2006). The non-cellular components include agglutinins, precipitins, natural antibodies, growth inhibitors, cytokines and lytic enzymes, as well as numerous antimicrobial peptides (AMPs) that mount a non-specific defense against invading pathogens.

The AMPs are structurally diverse as exemplified by the defensins. These cationic, cysteine-rich molecules fall into specific classes with recognizable core motifs. Conformational variants are formed based on both the number and orientation of their

specific α-helix or β-sheet (2 or 3) motifs (Hoffman and Hetru 1992, Ganz and Lehrer 1995, Hancock and Lehrer 1998). Some classes lack the α-helix altogether. Examples include the α-defensins and the θ-defensins (Tang et al 1999) that are cyclic dimers of truncated α-defensins ligated head-to-tail. Where present, however, the α-helices are invariably stabilized by three to four disulfide bridges. These various AMP classes are evolutionarily conserved and are found in organisms as diverse as plants, (Thomma et al 2002), mollusks (Hubert et al 1996, Seo et al 2005), insects and humans (Hiratsuka et al 1998).

The primary function of AMPs is to establish a rapid and sustainable response to a wide array of pathogenic challenges. Besides the broad spectrum of protection offered by AMPs, they are also energetically cost effective to synthesize. It is estimated that producing a single large immunoglobin (Ig) G molecule compared to 20 typically sized AMPs, may require 100-fold more energy (Barra et al 1998, Hultmark 1994, Otvos 2000). This energy efficiency in part accounts for why innate immune responses mediated through AMPs are considerably more rapid for an initial infection than can be mounted using adaptive immunity.

The AMPs may be constitutively expressed (Silphaduang and Noga, 2001) or induced by pathogenic challenge (Hiratsuka et al 1998; Shike et al 2002). Cecropin, the first inducible AMP to be isolated, was found in bacteria-challenged diapausing pupae of the giant silk moth (*Hyalophora cecropia*) (Hultmark et al 1982). Other cecropins were later isolated from other insects. Abaecin, for example, is a proline-rich AMP induced by bacterial challenge in honey bees (Casteels et al 1990). In some instances, AMPs are derived from cleavage products of larger proteins, which have unrelated functions (Boman 1995, Park et al 1998,

2000, Parish et al 2001). For a brief review on inducible antimicrobial peptides refer to Cociancich et al (1994).

Numerous antimicrobial peptides have been characterized in fish (see review in Noga and Silphaduang 2003), but no AMPs have yet been identified in channel catfish (*Ictalurus punctatus*), the most important aquacultured fish in the U.S. While searching for antimicrobial peptides in channel catfish, we discovered that fish exposed to either a bacterial infection or a very important protozoan parasite, *Ichthyophthirius multifiliis* ('ich'), had enhanced antimicrobial activity compared to healthy fish that had not been exposed to the infection. *Ichthyophthirius multifiliis* is a highly virulent ciliate that is one of the most important parasite infections of freshwater fish (Dickerson and Dawe 1995). It can readily infect healthy fish, often causing mortality within days to weeks of initial infection. We subsequently determined that this antimicrobial activity was mainly associated with the upregulation of a variant of the β-chain of hemoglobin (Hb-β), that at least one of the peptides derived from the Hb-β had potent and selective activity against ich, and that this sequence of Hb-β was highly expressed in the epithelial tissues of the skin and gill, which are the target sites for ich infection. This response represents a novel and possibly important new function for hemoglobin.

MATERIALS AND METHODS

Purification of Antimicrobial Activities

Spontaneously Infected and Healthy Fish

We sampled three separate groups of fish. First, we sampled five fish from a population of about 35 channel catfish (*Ictalurus punctatus*, 1.5-2 kg, 18-24 mo old) in summer 2003 that was recovering from a recent outbreak of *Ichthyophthirius multifiliis* (ich). Fish had been held in a 4000 l freshwater aquarium at the Pamlico Aquaculture Field Laboratory (Aurora, NC) when they developed a spontaneous infection of ich. Fish were sampled five weeks after the start of the outbreak, which was four weeks after a week-long treatment with formalin to treat the infection. All fish had apparently completely recovered (i.e., had no gross evidence of ich infection) at the time of sampling. We also sampled two fish from a population of 25 fish (0.8-1 kg, 12 mo old) in Fall 2003 that had been maintained in a 1200 l freshwater aquarium at North Carolina State University (NCSU) for over 10 mo without any evidence of disease.

We also sampled a third group of 28 channel catfish (1-1.5 kg, 12-18 mo old) in summer 2001 that had been held in a 1200 l aquarium at NCSU. These fish had recently recovered from an unidentified bacterial infection that had caused extensive, shallow skin ulcers on all of the fish. Four fish were sampled seven weeks after the start of the outbreak, which was five weeks after a two-week treatment with oxytetracycline-medicated feed. All fish had apparently completely recovered (i.e., eating and behaving normally, no gross evidence of skin lesions) at the time of sampling.

Preparation of Tissue Extracts

Gill extracts from all three groups of fish were prepared in the same manner, as described in Robinette et al (1998) and Noga et al (2001). Briefly, fish were anesthetized using Finquel® (Argent Chemical Laboratories, Redmond, WA) and maintained on ice throughout processing. Gill lamellae were removed and gently blotted to remove excess blood; the lamellae were then trimmed off the arches and placed into a beaker with boiling 1% acetic acid (HAc). The tissue was immediately diluted 1:4 (v/v) in boiling 1% HAc and boiled for five min, after which it was placed directly on ice.

The acidified tissue was then homogenized on wet ice for five min using a homogenizer (Polytron, Kinematica AG, Littau-Lucerne, Switzerland). The homogenate was centrifuged at 15,000 x g for 45 min and the supernatant was stored at -80°C until use.

Continuous Acid-Urea PAGE (CAU-PAGE)

Gill extract was run on a preparative continuous acid urea polyacrylamide gel electrophoresis column (CAU-PAGE)(Bio-Rad Prep Cell 491, Bio-Rad Laboratories, Richmond, CA) as described by Harwig et al (1993). A 6.5 cm high separating gel (12% acrylamide; 37.5:1 w/w acrylamide:bis solution) containing 5% HAc and 4.8 M urea was polymerized overnight in a 28 mm diameter tube using TEMED (0.48%, v/v) and APS (0.22%, w/v) as catalysts. No stacking gel was used. The gel was pre-run for 90 min at 4^{o}C at 40 mA with reversed polarity (lower chamber-cathode). Both the upper and the lower buffer chambers were filled with 5% HAc. After pre-running the gel, the upper buffer chamber was

refilled with fresh 5% HAc. Ten ml of acidified extract was mixed with 4 ml of sample buffer (3.0 M urea in 5% HAc) and clarified by centrifugation at 12,000 X g for 6 min. The supernatant was electrophoresed at 4°C, at 30 mA and eluted at a flow rate of 0.8 ml/min. Twelve ml fractions were collected over 12.5 hr (600 ml of eluate), stored at -70°C overnight and then lyophilized. After lyophilization, each fraction was dissolved in 100 µl of 0.01% HAc and 3 µl of each fraction was tested for antibacterial activity against *E. coli* D31 using a radial diffusion assay (see below).

Reversed Phase High Pressure Liquid Chromatography (RP-HPLC)

The fractions eluted off the CAU-PAGE that contained the most rapidly migrating antibacterial activity were pooled and lyophilized. After dissolving in 0.01% HAc and filtering through a 0.45 µM membrane filter, the pool was loaded onto a C_4 reverse-phase column (Jupiter 5 µm, 300 Å, 4.6 x 250 mm, Phenomenex, Torrance, CA, USA). The sample was eluted at a flow rate of 1 ml/min beginning with an isocratic gradient of 7% acetonitrile in 0.1% TFA for 4 min followed by a linear gradient of 7-70% acetonitrile in 0.1% TFA for 55 min and then another isocratic elution for 5 min using 70% acetonitrile in 0.1% TFA. The eluate was monitored at 214 nm using a Waters 486 UV detector. Fractions were collected by hand, lyophilized, dissolved in 0.1% TFA, and tested for activity against *E. coli* D31 via the RDA. Peaks having activity were recycled through the column under the same conditions.

Radial Diffusion Assay (RDA)

Antibacterial activity during purification was assessed with the radial diffusion assay (Robinette et al 1998). Briefly, an overnight culture of *Escherichia coli* D31 grown at 37°C

in trypticase soy broth with 1% NaCl was washed 3 times by centrifuging at 590 x g for 10 min at 4^{o}C followed by resuspension with cold, 0.1M phosphate-buffered saline (pH 7.0). The bacterial suspension was adjusted to an optical density (OD_{570}) of 0.1 (10^8 CFU/ml). One ml of the bacterial suspension was added to autoclaved Luria-Bertani broth containing 0.5% NaCl, 200 mM phosphate buffer (pH 6.7), 1.5% low EEO agarose and 100 µg/ml of streptomycin sulfate, that was at 48^{o}C in a water bath. Ten ml of the bacterial suspension was poured into sterile 100 cm^2 Petri dishes with a 10 mm grid. 3 µl of sample was pipetted into 2.5 mm diameter wells for RDA, and the plate was incubated at 37^{o}C for 18 hr, at which time clearing zone diameters were measured to the nearest 0.1 mm. The amount of antibacterial activity recovered at each purification step was assessed by calculating the Units of activity from the clearing zone diameters (Noga et al 2002) by using a standard curve of a pure polypeptide antibiotic (calf histone H2B, Cat#223514, Roche Applied Science, Indianapolis, IN).

Discontinuous Acid Urea-Polyacrylamide Gel Electrophoresis (DAU-PAGE, 'Bug Blot')

Discontinuous acid-urea PAGE was used to monitor sample purity during purification and to characterize the antimicrobial response of fish to challenge with ich. Native gels (100 mm wide x 75 mm long x 1.0 mm thick) were prepared having 12% acrylamide; 37.5/1 (w/w) acrylamide/bis solution, 4.8 M urea, 5% acetic acid (HAc). TEMED (*N, N, N`, N`*-tetramethylethylenediamine, 0.48% [v/v]) and APS (ammonium persulfate, 0.22% [w/v]) were used as catalysts for gel polymerization. The gel was allowed to polymerize overnight

at room temperature. The APS and TEMED were removed by pre-running the gel on a mini-gel electrophoresis unit (Mini Protean II, Bio-Rad, Richmond, CA), with 5% acetic acid for 60 min at 150 V with reversed polarity (lower chamber-cathode). No stacking gel was used. Samples were first mixed (1:1) with sample buffer (3.0 M urea in 5% HAc with 0.01% methyl green) and electrophoresed with 5% HAc at 150 V for 50 min at room temperature. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 and de-stained in MeOH:HAc:H_2O (4:1:5).

The crude tissue extract as well as the semi-purified fractions were run on duplicate gels or in hemi-gels to assess their apparent molecular mass and purity (Coomassie stained gels), as well as to compare their antibacterial activity by a modified antibacterial AU-PAGE gel overlay assay (Lehrer et al 1991). Briefly, samples were run either on duplicate gels or run in duplicate on hemi-gels and one gel (or hemi-gel) was stained as described above. The duplicate gel was rinsed twice, for 15 mins each time, in 100 mM phosphate buffer (pH 7.4). The gel was then placed on an *E. coli* D31 bacterial plate prepared as described for the RDA procedure and incubated at 37ºC for 3 h to allow the proteins to diffuse into the agarose. The bacterial plate was returned to 37ºC for overnight incubation (18 - 20 h) and observed for clear zones where the growth of bacteria was suppressed by the protein bands with antibacterial activity.

Structure Determination and Peptide Synthesis

Electrospray ionization mass spectrometry (ESI-MS) data for all AMP isolated were obtained using a quadrupole time-of-flight (Q-TOF) mass spectrometer (Waters/Micromass,

Manchester, UK). A small aliquot of the purified peptide was diluted in 1% formic acid/50% acetonitrile/water to approximately 5 pmole per µl to prepare for injection into the mass spectrometer. The aliquot was injected using a nanospray ionization source equipped with a PicoTip Emitter (#BG10-78-4-CE20, New Objectives, Woburn, MA). Mass spectral data were obtained in the positive mode and transformed to the average molecular mass using the MaxEnt algorithm. Automated Edman chemical degradation of the intact purified peptide was performed according to the manufacturer's protocol on a Procise 494 cLc Peptde/Protein Sequencer (Applied Biosystems, Foster City, CA) equipped with an on-line HPLC system.

Homology searches of the purified peptides were performed using BLASTP 2.2.10 and TBLASTN 2.2.10 on Genome Net (http://www.ncbi.nlm.nih.gov/BLAST). The calculated isoelectric point (pI) and molecular mass of the sequenced peptide were determined via ExPASy (http://www.expasy.ch/tools/peptide-mass.html). Secondary structure prediction software of Meiler et al (2002) was used for peptide modeling; determination of amphipathicity of predicted helical regions was carried out using the Pepwheel tool (EMBOSS GUI v.2.2.0, http://bioinfo.hku.hk/EMBOSS/).

The peptide representing the predominant antimicrobial activity in the gill extract (i.e., with the strongest antibacterial activity against *E. coli* D31 in the RDA) was synthesized via Fmoc chemistry on a Rainin Symphony instrument that provides on instrument cleavage of the peptide from the resin. After synthesis, the peptide was purified via analytical reverse phase HPLC using a YMC C-18 column (4 mm x 50 mm, 3 micron particle size, 120 angstrom pore size support) using an acetonitrile gradient that was eluted at 1 ml/min where buffer A was 0.05% TFA in water and buffer B was 80% acetonitrile in 0.05% TFA in water. The peptide was detected by its absorbance at 210 nm. Mass spectrometry of an aliquot of

purified peptide was carried out on a Micromass TofSpec SE mass spectrometer that was operated in positive ion mode and that is equipped with a nitrogen laser (337 nm), a reflectron, delayed extraction and a post acceleration detector. The purified peptide was lyophilized from 0.05% TFA/acetonitrile solution and stored desiccated under argon gas until reconstitution in solvent.

The RP-HPLC absorbance peak of 1 μg of synthetic peptide having the predominant antibacterial activity was used to determine the amount of native peptide in lyophilized RP-HPLC purified fractions. The height of the RP-HPLC absorbance peak for a known weight of synthetic peptide was used to estimate the amount of each native peptide by direct comparison of relative absorbance. The activity of native versus synthesized peptides were compared by performing serial dilutions of each peptide in the RDA.

Antimicrobial and Hemolytic Testing

Antibacterial Activity

The peptide with the strongest antibacterial activity against *E. coli* D31 in the RDA was further tested using a broth microdilution assay (Silphaduang and Noga 2001). Briefly, mid-logarithmic phase cultures in trypticase soy broth (TSB)/1% NaCl, Mueller Hinton Broth (MHB) or Luria Bertani Broth (LB) were washed and adjusted to 10^8 cells/ml as described for the RDA. The suspension was then diluted in MHB to 10^7 cells/ml and 100 μl was added to duplicate wells of a 96-well polypropylene microtiter plate (Costar, Corning Inc., Corning, NY).

Serial dilutions of peptide suspended in 0.01% HAc were diluted in 0.2% BSA in 0.01% HAc at 10 times the desired test concentrations and 11 µl of each peptide dilution were added to duplicate wells with the last column of wells having the diluent control. The plate was sealed with sealing tape (Corning #430454), incubated at 37ºC for 24-30 h, and then read using an ELISA plate reader (Elx800, Bio-Tek Instruments, Inc., Wivooski, VT) after growth was observed in the control wells. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic at which growth was reduced by more than 50%. The wells having the three lowest antibiotic concentrations and no visible growth were plated out on MHA in triplicate to determine the minimum bactericidal concentration (MBC). Plates were incubated at 37ºC for up to 24 h and MBC was determined as the lowest concentration at which a colony count equal to or less than 0.1% (= 10 colonies) of the CFUs contained in the original inoculum was observed.

Antiparasitic Activity

Antiparasitic activity was tested against two fish-pathogenic ciliates. *Ichthyophthirius multifiliis* feeds as a trophont within the skin and gill epithelium. When it matures (4-10 days under optimal temperature: 22°-25°C), the trophont exits the host, falls to the substrate and forms the dividing stage (tomont), which produces 800 free-swimming theronts that can immediately infect a new host (Dickerson and Dawe 1995). Ich was maintained in a channel catfish fingerling population by regularly adding naïve fish to an aquarium having infected fish. *Tetrahymena pyriformis* does not have tomont or theront stages and is transmitted

directly from fish to fish. An axenic isolate (#13-1620, Carolina Biological Supply Co., Burlington, NC) was maintained in *Tetrahymena* medium (#13-2315).

Ich trophonts were obtained by scraping 40-50 parasites off the skin of infected fish and transferring them to two ml of filter sterilized aquarium water in a polystyrene Petri dish. They were used immediately for testing. Tomonts were obtained by incubating trophonts in aquarium water for 2 hr, when they differentiated into tomonts. Theronts were produced by adding three trophonts to 500 µl of filter-sterilized aquarium water at 25°C. After 24 hr, the excysted theronts were used immediately.

Test wells of a 96-well flat-bottom polystyrene micro-titer plate (Costar, #3208) were loaded with 80 µl of filter-sterilized aquarium water and then 10 µl of each peptide dilution was added as described for antibacterial testing. Piscidin 1 was used as a positive control and diluent (0.2% BSA in 0.01% HAc) as a negative control. Using an inverted phase contrast microscope (Nikon, Tokyo, Japan), parasites were gently pipetted into wells containing the test solutions. Three trophonts, three tomonts or five theronts in a total volume of 10 µl of aquarium water were added to duplicate wells. Observations were made every min for the first 15 min, every 10 min for the next 30 min and then every 15 min for the following 75 min. Test plates were observed every hour for the following four hr and then every 24 hr to check for any changes in morphology or development of surviving parasites.

For testing *T. pyriformis*, the cultured stock suspension was diluted 1:20 with sterile water. After gently vortexing at low speed, the suspension was counted and adjusted with water to a final concentration of 15–20 parasites per 10 µl. Ten µl peptide dilutions were added to duplicate wells of a flat-bottomed 96-well polystyrene plate having 80 µl of filter-

sterilized aquarium water. Ten µl of the *Tetrahymena* suspension was then added to each test well and observations were made as described earlier for ich.

Parasites were observed for hyperactivity (sudden, rapid and random movement), decrease in ciliary movement and finally, death (defined as cessation of all ciliary movement; this was always accompanied by lysis). We also observed attempted encystment (i.e., presumably attempted tomont formation) of some ich trophonts. This was evidenced by secretion of the mucoid capsule or "cyst", which is characteristic of the tomont formation (Dickerson and Dawe 1995). When encystment occurred, it was always followed by excystment (emergence of the parasite from this developing cyst) and subsequent death.

The effect of physiological salt concentration on ich trophonts was also tested by performing the same assay in sterile Hank's Balanced Salt Solution (HBSS) instead of filter-sterilized aquarium water. Trophonts were scraped off the skin of infected fish and transferred to two ml of HBSS in a polystyrene Petri dish for testing. Parasites were then added to duplicate wells having peptides diluted in HBSS.

Hemolysis Assay

Channel catfish blood was collected by caudal venipuncture, diluted 1:1 (v/v) in Alsever's solution, and washed thrice in phosphate buffered saline (PBS, 50 mM sodium phosphate, 150 mM NaCl, pH 7.4) by centrifuging for one min at 2,700 x g at 4°C. The pellet was resuspended to a 10% suspension (v/v) in PBS and 50 µl of suspension was added to replicate wells of a 96-well polypropylene microtiter plate. Two-fold serial dilutions of peptide were prepared in PBS (0.2% BSA) and 50 µl of each dilution were added to duplicate

wells. A 0.1% solution of Triton-X 100 in water was used as the 100% hemolysis control. Piscidin 1 and magainin 2 were used as additional controls. After incubation for 10 min at RT, the plate was read at 490 nm in a plate reader (Elx800, Bio-Tek Instruments, Inc., Winooski, VT).

Upregulation of Hemoglobin-β Related Polypeptides by Parasite Challenge

Controlled Challenge with Ich

Eighteen channel catfish fingerlings (4"-6", 9-14 cm, 4-6 mo old) obtained from a commercial supplier were acclimated for five days to two 40 l aquaria (nine fish per aquarium) having biological filters. One aquarium served as unchallenged control while the other was used to challenge fish with ich. During acclimation, both aquaria were treated identically with respect to water changes, water quality testing, and feeding (fed twice daily with a commercial pelleted feed). During the experiment, fish were fed once daily and no water changes were made. Three fish were sampled from each aquarium immediately before the challenge was begun for time zero (T_0) baseline sampling (see *Collection of Tissue Samples*). Fish from the unchallenged aquarium were always sampled first to prevent any possible cross-contamination once the fish were challenged.

After all six fish (3 fish x 2 aquaria) were sampled at T_0, one heavily infected catfish fingerling (>200 trophonts on skin) was added to the challenge aquarium. The infected fish was removed after 48 hr, allowing enough time for the trophonts to detach from the host and develop into infective theronts. All fish were observed twice daily for evidence of ich

infection (presence of small white foci on the skin). Three fish from each aquarium were again sampled at 7 d (T_7) and 14 d (T_{14}) after challenge

Collection of Tissue Samples

At each sampling period, fish were euthanized one at a time and sampled as follows: Infection intensity was assessed by excising 1/4th of the first gill arch on the left side and scraping off all the epidermis (2 cm x 1 cm area) starting 3 cm from the base of the caudal fin on left side using a scalpel blade. The presence of ich trophonts was confirmed using an inverted phase contrast microscope. Untouched epidermis on the left flank and a corresponding region on the right flank were scraped off, obtaining exactly 50 µl of tissue that was placed into 3 volumes of 1% HAc with PIC and immediately boiled for 5 min. A 50 µl sample of gill tissue was similarly collected. After homogenizing the skin and gill samples with a Teflon-tipped manual homogenizer (Kontes #749516-0500), they were centrifuged at 15,000 x g for 10 min at 4°C and the supernatant was stored at -80°C until testing via DAU-PAGE and western blotting. A sample of each extract was also tested for antibacterial activity using the RDA. The Units of activity were calculated for each skin and gill sample. The PIC alone in 1% HAc was also tested for antibacterial activity.

Cellular Localization of Antibiotic Activity

Immunocytochemistry

Affinity purified rabbit anti-HbβP-1 antibodies were prepared against the conjugated peptide. Two milligrams of synthesized peptide was conjugated to keyhole limpet hemocyanin (KLH) using maleimide chemistry, which linked the peptide to KLH via a cysteine added to the N-terminal alanine. Immunogen was mixed with Complete Freund's Adjuvant (1:1) and KLH-conjugated peptide was injected into two New Zealand white rabbits at 5 subcutaneous sites (0.2 mL per site, 100 µg/injection) on days 0, 14, 28 and 42. Thirty ml of antiserum was collected from each rabbit on days 35 and 45. The antiserum was pooled and was then affinity-purified using the peptide conjugated to cyanogen bromide-activated agarose as an immunosorbent (10.5 mg of CLP was reacted with 15 g of agarose). One hundred and twenty ml of antiserum (two 30 ml bleeds from two rabbits) was loaded onto the column (Uniflow 4, Sterogene, Carlsbad, CA). After washing, the affinity-purified antibody was eluted and concentrated.

This method produced greater than 0.1 mg of peptide-specific antibody per mL of antiserum, as determined by recovered affinity-purified antibody. Antibody was greater than 95% IgG, as determined by immunoelectrophoresis using antibodies specific for rabbit IgG, IgM and serum proteins. The titer of the antibody was determined via ELISA, using pure peptide as the antigen coated onto a microtiter plate. Pure peptide (10 µg/mL in PBS, pH 7.2-7.5) was coated onto a microtiter plate at room temperature for 1 hr. The plate was then washed and post-coated with 1% BSA in PBS for 30 min. The plate was washed and then

dilutions of antibody in 1% BSA/PBS/0.01% Tween 20 were added, beginning at 1 µg antibody/mL. After incubation for 1 hr, the plate was washed, followed by addition of peroxidase-conjugated goat anti-rabbit IgG (h&l) in 1% BSA/PBS/0.01% Tween 20. After incubation for 1 h, the plate was washed and peroxidase substrate was added, incubated for 15 min, and then stopped with 1 N HCl (1:1). The absorbance was then read at 450 nm. The titer was read as the reciprocal of the antibody dilution (dilution of a 1 mg/mL solution) that produced a net optical density of 1.0, compared to a blank (non-coated well), which had an OD < 0.1. The titer of the antibody used in all assays was 1:400,000. The peptide-specific antibody had less than 1% cross-reactivity by ELISA, where 1% cross-reactivity is 100 times more antibody than is required to produce the same optical density with either free KLH, conjugated KLH, or free peptide that shares less than 3 amino acids in the sequence.

After fixing tissues for 24 hr, they were rinsed in deionized water and stored in 70% ethanol until immunochemistry testing. Histological sections were prepared routinely (Silphaduang and Noga 2001). The deparaffinized gill and skin tissue sections were blocked with goat serum for 30 min followed by treatment with the unlabelled specific antibody or non-immune rabbit serum in PBS with carrier protein and preservative (Biogenex) for 1 hr. The sections were then incubated in biotinylated goat anti-rabbit antibody for 1 hr after a brief wash in PBS and then similarly rinsed again after treatment. Sections were then treated with the developing reagent to visualize the specific antibody localization in the cells. All sections were evaluated blindly.

In-situ Hybridization

Probe Design

A homology search using the amino acid sequence was performed using BLASTp and tBLASTx v2.2.10. Identified cDNA sequences were used to locate base substitutions within the sequence that would result in the amino-acid substitutions observed in our sequenced AMP. The resulting sequence was searched once again in the Genbank EST database BLASTest v2.2.10 to identify putative mRNA transcripts for our AMP. Based on the hits in the EST database, PCR primers were designed to amplify the more promising genes (using the genomic template) that may fall in the hemoglobin β-chain gene family and transcribe HbβP-1. These primers were then used to detect the specific mRNA isolated from the catfish tissue collected from fish challenged with *I. multifiliis* and control, uninfected fish.

Genomic DNA was extracted from cells of frozen skin and gill tissue using either QIAmp Tissue Kit (Qiagen, Valencia, CA) or the Mo Bio Soil Extraction Kit (Mo Bio Laboratories, Calsbad, CA) according to the manufacturer's protocol. The genomic DNA sequence encoding for the entire putative hemoglobin β-chain sequence was PCR-amplified using the Catfish2UF forward (5′-ATGGTTCATTGGACAGACGC-3′) and the Catfish2R reverse (5′-GCACATCTTTATATTATGATGCG-3′) primers with an expected amplicon size of ~670 bp. Amplification was carried out using the GeneAmp® Gold PCR Reagent Kit (Applied Biosystems, Foster City, CA). The reaction mix contained ~10 ng of catfish genomic DNA from skin or gill, 10X buffer pH 8.3, 0.5 U AmpliTaq Gold polymerase (Applied Biosystems), 2.5 mM of each dNTPs, and 100 ng of each primer in a final reaction

volume of 50 µl. Amplifications were carried out in an iCycler® (Bio-Rad®, Richmond, CA) using the following conditions: 5 min at 95°C followed by 40 cycles (45 sec at 95°C, 45 sec at 50°C, 1 min at 72°C), with a final extension of 5 min at 72°C. Amplicons were cloned into plasmid vector pCR2.1® (Invitrogen®, Carlsbad, CA) using the TA Cloning Kit® following the manufacturer's protocol and the resulting plasmids were then sequenced on an ABI377 DNA sequencer using the Deoxy™ Terminator Cycle sequencing kit (Applied Biosystems – ABI™, Foster City, CA). Sequencing was performed following the manufacturer's instructions and using approximately 600 ng of plasmid DNA per reaction.

The probe for *in situ* hybridization was designed based on the sequence analyses of 11 clones obtained from the PCR amplicons. The clone sequences were compared with the cDNA sequence for *I. punctatus* Hb-β from Genbank (gi:38606322) and analyzed using the ClustalW Multiple Analysis tool as well as pairwise alignment (using optimal GLOBAL alignment) available through the BioEdit Sequence Alignment Editor (Hall 1999). A highly conserved region corresponding to the 5' end of exon-3 was identified and the reverse complement of the sequence used to design the probe. The probe sequence (5' - TTGCGGCCAGAGTTACGGT – 3') was synthesized and digoxigenin-labeled at the 3' end (GreenStar*™ Hyperlabeled Oligonucleotide Probe pack, GeneDetect.com Ltd., Bradenton, FL).

Hybridization

Paraffin-embedded gill and skin tissues were deparaffinized and rehydrated before quenching in 0.3% H_2O_2 in methanol for 20 min to eliminate endogenous peroxidase

interference. Quenching was followed by a 5 min rinse under running water before the slides were treated with the appropriate probe dilution (0.625 ng/ml) and incubated at 40°C for 1 hr. Hybridization was followed by detection using the GenPoint ™ Tyramide Signal Amplification System (DakoCytomation, Inc., Carpinteria, CA) with two stringency washes in 1X SSC and 0.5X SSC for 5 min each at 45°C and rinsing in Tris buffered saline with 0.5% Tween-20 (TBST) thrice for 5 min. The slides were then treated with polyclonal rabbit anti-DIG/HRP labeled antibody at 1:100 concentration (0.001mol/L) for 15 min followed by three rinses in TBST (5 min) before treatment with the biotinyl tyramide amplification reagent (15 min). This was followed by three rinses in TBST and 15 min incubation in secondary streptavidin-HRP reagent. The DAB chromogen used to detect the signal was diluted 1:50 for treating the slides after rinsing them once again in TBST (3 times, 5 min each wash). When the signal was detectable, the chromogen reaction was stopped by immersing slides in water for 1 min followed by several changes of water to remove residual chromogen. Slides were counterstained with hematoxylin and mounted in Permount.

RNase treated tissue sections were used to verify absence of any detectable signal and hence confirm binding of probe to the transcribed mRNA. Slides were incubated in 0.006% RNase (#R4642, Sigma-Aldrich, MO) dissolved in 20 mM Tris (ph 7.5), 1 mM EDTA at 37°C for 1 hr, then rinsed in TBST thrice for 5 min before proceeding to hybridization with probe. Binding specificity was further verified by treating duplicate slides with a control sense-probe (5' - ACCGTAACTCTGGCCGCAA - 3')(5 ng/ml) under identical hybridization conditions to that used for the anti-sense probe to assess degree of non-specific binding.

Statistical Analyses

Analysis of antibacterial activity in infected versus uninfected fish was compared with Student's t-test to verify significance using Analysis of Variance. Comparison of time to lysis of trophonts after treatment with AMPs was performed using Tukey's multiple pairwise comparison method for five concentrations (200 – 12.5 μg/ml) of each peptide. Measurements of anti-trophont activity were replicated four times (i.e. 4 duplicate wells) for each set of factors. All analyses were done using PROC GLM in SAS version 9.1 (SAS Institute, Cary, NC).

RESULTS

AMP Purification and Identification

The gill extract from channel catfish previously infected with *I. multifiliis* had much greater antibacterial activity than the pooled gill extract from healthy, uninfected fish (Fig. 1.1). We observed a similar response when we sampled fish that were recovering from a bacterial infection (Fig. 1.1 inset). Separation via CAU-PAGE demonstrated an uninterrupted range of antibacterial activity from fractions 11 to 34, with an activity peak at fraction 16 (Fig. 1.1). This fraction 16 peak migrated faster than the fractions that contained histone-like proteins (HLPs), polypeptide antibiotics that had previously been isolated from skin of channel catfish (Robinette et al 1998) and gill of rainbow trout (*Oncorhynchus mykiss*) and hybrid striped bass (*Morone saxatilis* x *M. chrysops*)(Noga et al 2001). The rapidly migrating activity peaks (fractions 11-16) were pooled and subjected to C_4 RP-HPLC. At least 23 peaks had antibacterial activity, but the peaks with the strongest activities migrated at 54.5%, 52% and 47.5% acetonitrile concentrations (in decreasing order of activity). These peaks were designated Peaks 1, 2 and 3, respectively (Fig. 1.2). Recycling all three peaks yielded a major peak at an identical elution point to that of the loaded sample and a minor peak (height $<1/16^{th}$ of the major peak) eluting at 57.5% acetonitrile. The minor peak did not show any antibacterial activity and was determined to be non-proteinaceous by electrospray ionization mass spectroscopy (ESI-MS) (data not shown). The other peaks having antibacterial activity were not identified.

The ESI-MS of the purified peptides yielded average masses of 3700.0 Da (Peak 1), 2231.3 Da (Peak 2) and 3423.0 Da (Peak 3) (Fig. 1.3). Complete sequencing of Peak 1 and Peak 2 by Edman degradation indicated that Peak 1 consisted of 33 residues while Peak 2 was a 19-mer fragment of Peak 1. A search using BLASTP 2.2.10 revealed that the 33-mer peptide had high homology (32 of 33 residues) with the C-terminus of channel catfish Hb-β (Suzuki and Nishikawa 1996, Yeh and Klesius 2003). Peak 1 corresponded to residues 116-148 of channel catfish Hb-β with a single residue substitution (lysine for asparagine) at the position corresponding to residue 118 (residue 3 in Peak 1) (Fig. 1.4). Peak 2 was the C-terminus (residues 15-33) of Peak 1, corresponding to residues 130-148 of Hb-β-chain. Peak 3 was partially sequenced with only the first eight N-terminal residues determined by Edman degradation. However, the observed mass (3423 Da) was in excellent agreement with the predicted mass of a 30-residue segment corresponding to residues 2-31 of Hb-β (Genbank Accession #AAR25199.1). Thus, we named Peaks 1, 2 and 3 as HbβP-1, HbβP-2 and HbβP-3, respectively.

Based on the absorbance of synthetic HbβP-1, we recovered 8 μg of HbβP-1, 2 μg of HbβP-2 and 16 μg of HbβP-3 per gram of gill tissue. The native and synthetic HbβP-1 peptides had identical lethal concentrations for *E. coli* D31 (6.3 μg/ml, 1.7 μM).

Antimicrobial and Hemolytic Activity

We synthesized HbβP-1 because it had the strongest antibacterial activity of all peaks. The next two most potent peptides (HbβP-2 and HbβP-3) were not tested further. While HbβP-1 had moderate activity against a few gram-negative bacteria, including fish pathogens

(*A. hydrophila* and *V. alginolytics*; MICs of 12.5-50 μg/ml), it had no activity against most bacteria tested, including all gram-positive isolates (Table 1.1).

However, HbβP-1 activity against ich trophonts (Table 1.2) was comparable to that of the most susceptible bacterial pathogens (Table 1.1), with very rapid and in some cases almost instantaneous effect. Within 2-4 min exposure to 25 μg/ml of HbβP-1, trophonts became hyperactive and lysed. Exposure to 25 μg/ml of piscidin 1 also caused lysis, but took significantly longer, at 7-8 min ($p<0.0001$). HbβP-1 lysed some trophonts at as low as 6.3 μg/ml (1.7 μM) (Fig. 1.5), after 6-8 min. Exposure to a wide concentration range (6.3-50 μg/ml) of HbβP-1 caused most trophonts to encyst and subsequently excyst (Fig. 1.5) prior to lysing. Encystment/excystment was never seen in trophonts exposed to piscidin 1. HbβP-1 was highly selective for the trophont stage, having no activity against either the infective theront or the encysted tomont (Table 1.1). Piscidin 1 was also ineffective against tomonts but killed theronts at as low as 6.3 μg/ml.

In physiologic salt concentrations, HbβP-1 was still highly cidal to trophonts, with an MLC only one dilution lower than that in freshwater; some parasites were still killed in as low as 6.3 μg/ml of HbβP-1. However, in HBSS, trophont death was delayed, occurring after 75-105 min. Also, trophonts were never observed to encyst/excyst. Trophonts in HBSS alone survived for two to six hr, while those in filter-sterilized freshwater differentiated into tomonts within two hr and formed viable theronts overnight.

HbβP-1 also lysed all *T. pyriformis* cells within 2-3 min of exposure at 25 μg/ml (Fig. 1.5); piscidin 1 required almost twice as long (4-5 min)($p<0.0001$) to kill all parasites at this concentration. In contrast to its effect on protozoa, HbβP-1 had no detectable hemolytic activity against channel catfish erythrocytes, even at the highest concentration tested, 400

μg/ml. This contrasts with piscidin 1, which had some hemolytic activity starting at 12.5 μg/ml (Fig. 1.6).

Expression of Polypeptides Derived from Hemoglobin-β after Experimental Ich Challenge

Antibacterial Activity

The mean antibacterial activity in the skin extracts of fish sampled from the two aquaria at time 0 (T_0) was not significantly different (6.4 U and 5.9 U, $p<0.005$, N = 3 fish per aquarium). At seven days post challenge (T_7), the mean antibacterial activity in skin extracts of fish challenged with ich (9.9 U) was 74% greater than that of unchallenged fish (5.7 U). At T_{14}, the mean activity in skin of challenged fish (14.9 U) was 140% greater than the control fish (6.2 U). The activity in infected fish was significantly greater than that in uninfected fish ($p<0.005$) at both T_7 and T_{14}.

The mean antibacterial activity in the gill extracts of fish sampled from the two aquaria at time 0 (T_0) was not significantly different (18.0 U and 17.2 U, $p<0.005$, N = 3 fish per aquarium). The mean antibacterial activity in gill extracts of fish challenged with ich (23.2 U) was 52% greater than that of unchallenged fish (17.7 U) at T_7. At T_{14}, the mean activity in challenged fish (41.4 U) was 172% greater than the control fish (15.2 U). The activity in infected fish was significantly greater than that in uninfected fish ($p<0.005$) at both T_7 and T_{14}. PIC alone in 1% HAc had no activity.

Skin and gill extracts from control and ich-challenged fish were pooled and tested for activity using the DAU-PAGE bug blot. All extracts (including unchallenged controls) had a very strong activity zone that corresponded to the migration rate of calf histone H2B. This zone is known to have a high concentration of histone-like proteins (HLPs), with strong antibacterial activity (Robinette et al 1998, Noga et al 2001). While the tissue extracts collected at T_7 did not show detectable increase in activity on the bug-blot (Fig. 1.5), both skin and gill extracts from ich-challenged fish had strongly increased antibacterial activity at T_{14}. The majority of this increased activity was more rapidly migrating than HLPs, and included the zone where pure HbβP-1 migrated (Fig. 1.5). The HLP region also appeared to show increased activity in the challenged fish (T_{14}).

Immunochemical Response

Immunocytochemistry of skin and gill from challenged fish showed a strong signal with anti-HbβP-1 antibody, especially in the damaged region immediately surrounding the parasites residing in the epithelium (Fig. 1.8G). Most of the antibody binding in the skin appeared to be in the epithelium and in alarm cells (Fig. 1.8). However, some damaged regions of the tissue sections did not show a strong signal if there was no parasite present in the section. This antibody also gave a very strong signal within erythrocytes (Fig. 1.8).

In Situ Hybridization

Hybridization of the *in situ* probe was detected especially within the skin epithelial cells as well as in the alarm cells (Fig. 1.9). Most signal was detected in injured areas. Some damaged areas did not show strong probe binding as was also seen in the immunocytochemistry results. The probe bound very strongly within the gill epithelium and not in erythrocytes. Lack of signal after treatment with the sense-probe indicated sequence-specific binding of the antisense-probe.

DISCUSSION

Our study demonstrates that peptides homologous to Hb-β are upregulated in skin and gill epithelium of channel catfish after infection with the important parasite *Ichthyophthirius multifiliis*. The 33-mer HbβP-1 varied by a single amino acid from the published sequence of channel catfish Hb-β (Genbank accession gi:38606322). This is most likely explained by these peptides being derived from a variant of Hb-β rather than from an unrelated protein having homology to Hb-β. In most species, multiple genes encode different polypeptide chains that form hemoglobin variants (Bunn et al 1986). For example, there are two known variants of Hb-β in rainbow trout (Brunori et al 1975; Lau et al 1975) and two variants of Hb-β in the armored catfish (*Liposarcus anisitsi*) that include anodic and cathodic hemoglobins differentiated on the basis of their electrophoretic mobility (Smarra et al 2000). Human hemoglobin has 232 known Hb-β variants (Bunn and Forget 1986)

All of the upregulated AMPs (HbβP-1, -2 and -3) had antibacterial activity and one (HbβP-1) had potent, selective activity against ich and another fish parasite (*Tetrahymena pyriformis*). A conservative estimation of the amount of HbβP-1 that we isolated from catfish gill tissue (8 μg/g) was within the concentration that was lethal to ich in vitro (Table 1.2). Most importantly, our calculations were very conservatively based upon an assumption of 100% recovery of HbβP-1 from the crude tissue extract, which is never achieved. It also does not take into account that the peptide is concentrated in only certain parts of the gill tissue and is not evenly distributed throughout the gill. This antiparasitic activity was little affected by physiological salt concentrations, including Na^{+}, Ca^{++} and Mg^{++}, all known to strongly

inhibit most AMPs (Bals et al 1998a, 1998b; Jenssen et al 2006). This further suggests the ability of HbβP-1 to function extracellularly in the tissue.

The activity of HbβP-1 was stronger against parasites than against almost all bacteria tested (Tables 1, 2). HbβP-1 was also not lytic to channel catfish erythrocytes at the highest concentration tested (400 μg/ml, 108 μM)(Fig. 1.6), further suggesting anti-parasite specificity. The stage-specific activity of HbβP-1 also suggests an evolutionary selection towards targeting the parasitic (trophont) stage rather than the infective (theront) stage, which correlates with the much longer exposure time of the trophont to the host. This would allow HbβP-1 to be at an effective concentration for a much longer time in vivo, since the theront rapidly (within 40 min) differentiates into a trophont after infecting the fish (Dickerson and Dawe 1995). Also, the apparent induction of an avoidance response (i.e. hyperactivity) followed by encystment suggests that HbβP-1 might also induce the trophont to exit the host tissue and differentiate prematurely. Such trophonts would be expected to die once detached (since no trophonts survived peptide induced encystment/excystment in vitro), avoiding the need for gradual disposal of dead parasites that would otherwise have remained within host tissue. However, a more likely scenario may mimic the effects observed by Clark et al (1996) with a murine monoclonal antibody (mAb) directed against an *I. multifiliis* surface antigen (immobilization antigen). While the mAb immobilized trophonts in vitro, systemic (intraperitoneal) administration to channel catfish induced the premature exit of trophonts from the skin.

Stage-specific antiparasitic activity has also been reported for other polypeptide antibiotics. Histone-like protein 1 (HLP-1), a major antimicrobial polypeptide in the skin and gill of fish, is active only against the trophont stage of the dinoflagellate ectoparasite,

Amyloodinium ocellatum, with no activity against the tomont or infective (dinospore) stages (Noga et al 2001). Stage-specific antiparasitic activity has also been reported for magainin, cecropin and two arthropod defensins (isolated from the dragon fly, *Aeschna cyanea* and flesh fly, *Phormia terranovae*) against the malaria parasites *Plasmodium falciparum P. gallinaceum, P. cynomolgi,* and *P. knowles* (Shahabuddin et al 1998, Gwadz et al 1989). These AMPs were effective against the late oocyst and sporozoite stages of *Plasmodium* but not against the early ookinete stages. However, these in vitro studies were somewhat artificial since none of these peptides are naturally present in the mosquito vector.

The mechanism by which HbβP-1 exhibits anti-parasitic specificity is unknown. While it is cationic (pI 8.49) and appears to have some amphipathic structure, it does not appear to form a strong amphipathic α-helix like most other linear AMPs (Noga and Silphaduang 2003), which might account for its weak antibacterial activity. Based upon preliminary structure predictions, HbβP-1 probably forms an alpha-helical motif at residues 11-28 (Fig. 1.10) with a random coil at the flanking C and N-terminal residues (A. Ullal, J. K. Seo and E. J. Noga, Unpublished Data).

It has been known since the mid-20th century that the intact Hb molecule, consisting of four subunits (two α and two β), possessed antibacterial activity (Hobson et al 1958). At the time, this property was dismissed as not being biologically relevant. However, several studies have recently identified AMPs derived from in vitro (Parish et al 2001) or in vivo (Liepke et al 2003, Mak et al 2004) proteolysis of either Hb-α or Hb-β. Interestingly, some of these AMPs have preferential activity for eukaryotes. A 36-mer peptide derived from the C-terminus of Hb-β, isolated from human placental blood, had stronger activity against the yeast *Saccharomyces cervisiae* (MIC 12.8 μM) than against most bacteria tested (only two of

eight strains had an MIC <12 μM)(Liepke et al 2003). A homologous 31-residue fragment from human Hb-β also had potent activity against *Candida albicans* (MIC 3 μM), while MICs against gram-positive bacteria were 5 to 250-fold higher (Parish et al (2001). Parish et al (2001) also found that the intact Hb tetramer from an unidentified snake, as well as alligator (*Alligator mississippiensis*), horse and human had some antimicrobial activity. In addition, multiple fragments of Hb-α and Hb-β from human menstrual blood had antibacterial activity against *E. coli* (Mak et al 2004). A 32-residue Hb-β C-terminal fragment had much stronger activity against gram-positive and gram-negative bacteria compared to a 22-residue, Hb-α fragment. Interestingly, neither peptide tested had any activity againt *C. albicans* (>300 μM). Recently, Fernandes and Smith (2004) detected antibacterial activity in an acid extract of rainbow trout (*Oncorhynchus mykiss*) erythrocytes. Although not identified, SDS-PAGE showed a protein having molecular weight comparable to that of Hb-β (~16 kDa), as well as a possible dimer of Hb-β (~31 kDa). The crude erythrocyte extract and the eluate from solid phase C_{18} separation had multiple bands below the level of Hb-β on the gel, suggesting the presence of Hb fragments.

Many peaks in the RP-HPLC separation of gill extract from ich-exposed channel catfish had antibacterial activity (Fig. 1.1). While we only identified relatively weak antibacterial activity in the three peptides that we purified, some of the other unidentified polypeptides might have much stronger activity against other pathogens that we did not screen. In addition, this mixture of polypeptides might have much greater total antibacterial activity than its separate polypeptides. Histone-derived peptides from mucus and blood of coho salmon (*Oncorhynchus kisutch*), while having no in vitro antibacterial activity,

potentiated the activity of flounder AMP pleurocidin, chicken lysozyme, and extracts from the mucus and serum of coho salmon that contained lysozyme (Patrzykat et al 2001).

Combining different types of AMPs (β-sheets, amphipathic α-helices, loop peptides and extended peptides) can result in significant synergism (Yan and Hancock 2001). In some cases these peptides naturally coexist (e.g. the loop peptide bactenecin and the linear peptide indolicidin in bovine neutrophils). The advantages of such cooperative action are three-fold. First, individual peptides are preferentially induced at specific sites in the body. Second, different peptides cover gaps in the activity spectrum of other peptides expressed at such locations. And third, the cooperativity reduces the individual concentrations required to kill a pathogen (Yan and Hancock 2001). Peptide fragments of Hb-α and Hb-β, isolated from human menstrual blood, are synergistic against gram-positive and gram-negative bacteria (Mak et al 2004). It is well known that ectoparasite infections predispose fish to secondary bacterial infections (Bandilla et al 2006). Thus, the production of this multiple AMP arsenal, that not only has antiparasitic activity but also antibacterial activity, would allow protection against multiple invaders that are frequently present in an open wound of an aquatic animal. The likelihood that the Hb-β AMP response is directed at other pathogens in addition to parasites is also suggested by its strong upregulation in response to spontaneous bacterial infection (A Ullal and E Noga, Unpublished Data).

As mentioned previously, while it is possible that HbβP-1 and the other AMPs related to Hb-β might be coded by genes that are different from Hb-β, it is more likely that they are produced by proteolytic cleavage of upregulated Hb-β. We observed increased expression of a ~16 kDa protein in the skin of ich-challenged fish that presumably was Hb-β (A Ullal and E Noga, Unpublished Data); this is unlikely to be a blood contaminant since catfish skin has

no blood vessels (Grizzle and Rogers 1976). And recently, Hb-α and Hb-β were found to be constitutively expressed in alveolar epithelial cells of humans, rats and mice (Newton et al 2006). Proteins having the molecular weight of Hb-β and with an N-terminus homologous to Hb-β have also recently been identified in microsomes from gill epithelium of five species of primitive to advanced teleosts, including Mozambique tilapia (*Oreochromis mossambicus*), rainbow trout, common carp (*Cyprinus carpio*), European eel (*Anguilla anguilla*) and elephant fish (*Gnathonemus petersii*)(Schuurmans Stekhoven et al 2004). Microsomes, membrane-bound vesicles that are formed by fragmentation/budding from rough endoplasmic reticulum, are involved in intracellular protein transport to the Golgi complex for concentration and packaging into zymogen granules and then to the plasma membrane for secretion extracellularly (Jamieson and Palade 1964; Wells 2005). Microsomes may be transporting Hb-β via the Golgi complex and zymogen granules to sites of epithelial injury, where fragments of Hb-β may be generated within the microsomes or extracellularly. Based on the potential cleavage sites of channel catfish Hb-β (PeptideCutter, www.ExPaSy.org), HbβP-1, -2 and -3 could be generated by a combination of proteases such as endopeptidase K, trypsin and chymotrypsin. Proteolytic cleavage might also explain the numerous activity peaks in ich-exposed fish (Fig. 1.2), some of which might be other Hb-β fragments. However, other more specific enzymes or limited exposure to these enzymes would be needed to generate HbβP-1, -2 and -3, since continued exposure to these particular enzymes would be expected to result in further peptide cleavage and production of smaller peptides.

Generation of an AMP from a larger functional protein also occurs with parasin I, a 19-mer AMP that is formed from cleavage of histone H2A by cathepsin D. Cathepsin D is generated from procathepsin D by release of a metalloprotease during epidermal injury of

Asian catfish (*Parasilurus asotus*)(Cho et al 2002). Histone H2A is also cleaved by pepsin to form the AMP buforin I in the Asian toad (*Bufo bufo gargarizans*) (Kim et al 2000). Lactoferricin B is another well-known example of a proteolytic cleavage product of a larger molecule, lactoferrin that has greater antibacterial activity than the parent protein (Bellamy et al 1992, Fernaud and Evans 2003).

In the case of HbβP-1, another possibility is that the infecting pathogen (parasite or bacterium) might produce enzymes that are involved in production of the Hb-β peptides. For example, bovine Hb-α is processed into a 29-mer AMP in the gut of a tick (*Boophilus microplus*) after a blood meal on its cattle host. It has activity against eukaryotes (fungi) and gram-positive bacteria (Fogaca et al 1999). None of the Hb fragments that we observed in our tissue extracts are likely to result from artifactual proteolysis because the tissue was boiled in the presence of high concentrations of PIs to prevent this. Boiling alone was just as effective as boiling with the addition of PIs (A Ullal and E Noga, Unpublished Data).

In situ hybridization (Fig. 1.9) and immunohistochemistry (Fig. 1.8) clearly indicated that the Hb-β sequence corresponding to HbβP-1 is both encoded for and expressed within channel catfish skin and gill epithelial cells after ich challenge (Figs. 7-9). HbβP-1 or related AMPs might even enter trophonts (Fig. 1.8, 9). The antibody against HbβP-1 also most likely strongly cross-reacted with catfish Hb-β due to a 97% sequence homology with Hb-β; this was suggested by the strong antibody localization within erythrocytes (Fig. 1.8), which also served as confirmation that the antibody was binding to a Hb-β epitope. Our demonstration of this inducible extra-erythrocytic expression of HbβP-1 in tissues that are in intimate contact with ectoparasites (such as ich and *Tetrahymena*) further support its role as a first line of defense against infection. Although certain areas of epidermal damage did not show strong

immunoreactivity, this was most likely due to the focal and transient nature of ich infection, which might result in multiple, discrete sites of upregulation, which often might not be associated with the presence of the recently exited parasite. We also observed some immunoreactivity in clinically normal (unchallenged) fish, although the response was always very mild and limited compared to ich-challenged fish.

Increased antibacterial activity was detected in the RDA beginning at 7 days and in bug blots beginning at 14 d after challenge (Fig. 1.7), before any detectable specific immune response develops against ich (Clark et al 1988), which takes 3-4 weeks to develop (Burkart et al 1990). Thus, this innate response might allow time for the host to develop an adaptive, specific antibody response (Maki et al 2003; Xu et al 2002; Beckert et al 1964). Although expression levels beyond 14 days were not investigated, the increasing activity in the RDA over the 14-day infection suggests that AMP expression is sustained over 2 weeks and may be expected to continue past this time since strong expression of HbβPs was detected almost 5 weeks after the appearance of a spontaneous *I. multifiliis* infection (Fig. 1.5).

The presence of significantly more antibacterial activity in the region where HbβP-1 migrates on a bug blot (Fig. 1.7) was consistent with upregulation of HbβP-1. The diffuse activity surrounding the HbβP-1 migration zone is probably due to the presence of additional AMPs, some of which are probably products of Hb-β. Gill, being highly vascularized, was clearly contaminated with a significant amount of blood. Thus, a considerable amount of Hb-β that originates from erythrocyte Hb could affect interpretation of the activity in the zone where this protein would be expected to migrate (in the area of the HLPs). A strong clearing zone corresponding to the migration zone of HLPs (Noga et al 2001) was present in bug blots of both unchallenged and ich-challenged fish. However, whole blood of either unchallenged

or challenged fish did not display any antibacterial activity in the HbβP-1 region (Fig. 1.7). In addition, the upregulation of this large, rapid-migrating zone in the HbβP-1 region of skin was similar in intensity to that of gill (Fig. 1.7), suggesting that a significant response was present in the absence of significant blood contamination since in channel catfish of this size, the skin is avascular (Grizzle and Rogers 1976) and thus would have little to no blood contamination. Low levels of antibacterial activity, some of which co-migrated with HbβP-1, were also observed in some pooled tissue extracts of control (unexposed) fish (Fig. 1.7), which suggests possible low level constitutive expression of at least some of these AMPs.

While upregulation of AMPs in response to pathogenic challenge has been demonstrated against a number of microbes (Shike et al 2002, Cociancich et al 1994, Frohm et al 1997), there are only two studies documenting this response against parasites. Arthropod defensin mRNA is upregulated in the midgut of adult mosquitoes (*Anopheles gambiae*) that are experimentally fed *Plasmodium berghei*-infected mouse blood (Dimopoulus et al 1997). Both the mRNA coding for the AMPs and the peptides drosocin, diptericin and drosomycin, are upregulated in the hemolymph and gut of the fruit fly (*Drosophila melanogaster*), 24 hr after challenge with the kinetoplastid flagellate gut parasites *Crithidia bombi* and *C. fasciculate* (Boulanger et al 2001).

To our knowledge, our study is the first to demonstrate that any Hb sequence is upregulated in response to microbial or parasitic infection. However, a recent study has shown that exposure of murine macrophages to lipopolysaccharide in combination with IFN-γ induces the expression of Hb-β (Liu et al 1999). Based on Hb's ability to reversibly bind nitric oxide (NO) and transport it to tissues, Liu et al (1999) postulated that upregulation of Hb-β in macrophages may protect them from nitrosative stress during NO activation

following an infection. Although they demonstrated by western blotting that only activated macrophages expressed Hb-β, experiments measuring decay of NO in the presence of activated or resting macrophages showed no difference; thus, the role of this Hb-β upregulation was undetermined.

In summary, we demonstrated that antibacterial peptides homologous to hemoglobin-beta are upregulated in skin and gill epithelium of channel catfish after spontaneous or experimental infection. One of these AMPs has potent, selective, stage-specific activity against ich and is also lethal to another fish parasite (*T. pyriformis*). The strong antiparasitic activity of this Hb-β-derived AMP and the numerous other antimicrobial peptides expressed, suggests that this response might play an important role in innate immunity to many infections.

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Table 1.1. Antibacterial activity of HbβP-1 and piscidin 1. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of antibiotic at which growth was reduced by more than 50%. The minimum bactericidal concentration (MBC) was defined as the lowest antibiotic concentration at which growth was reduced by 99.9%. The MIC was identical to the MBC for all isolates (n = 2 replicates).

Bacterium	**Gram**	**MIC (µg/ml [µM])**	
		HbβP-1	**Piscidin 1**
Vibrio alginolyticus (88-5009-A2)	-	50.0 [13.5]	3.1 [1.2]
Escherichia coli (D-31)*	-	12.5 [3.4]	3.1 [1.2]
Aeromonas hydrophila (88-5009-C3)	-	12.5 [3.4]	3.1 [1.2]
Shigella flexneri (00-1402)	-	>200 [>54]	3.1 [1.2]
Pseudomonas aeruginosa (00-1400)	-	>200 {>54]	12.5 [5]
Edwardsiella ictaluri (91-3055)	-	>200 [>54]	-
Streptococcus faecalis (00-1403)	+	>200 [>54]	3.1 [1.2]
Streptococcus iniae (00-1417)	+	>200 [>54]	3.1 [1.2]
Staphylococcus aureus (00-1404)	+	>200 [>54]	3.1 [1.2]

Table 1.2. Activity of HbβP-1 and piscidin 1 against the protozoan fish parasites ich (*Ichthyophthirius multifiliis*) and *Tetrahymena pyriformis*. The minimum lethal concentration (MLC) was defined as the lowest antibiotic concentration at which all cells lysed, while the minimum inhibitory concentration (MIC) was defined as the lowest concentration where at least one parasite died (of 3 total) (n = 2 replicates i.e. 2 wells). Parasites were examined at regular intervals for 24 h or until death. All experiments were performed in freshwater unless noted otherwise.

	HbβP-1		**Piscidin 1**	
Parasite	**MLC (μg/ml) [μM]**	**MIC (μg/ml) [μM]**	**MLC (μg/ml) [μM]**	**MIC (μg/ml) [μM]**
Ich trophont	12.5 [3.4]	6.3 [1.7]	6.3 [2.5]	6.3 [2.5]
Ich tomont	>400 [>108]	>400 [>108]	>200 [>80]	>200 [>80]
Ich theront	>400 [>108]	>400 [>108]	12.5 [5]	6.3 [2/5]
Ich trophont in HBSS	25.0 [6.8]	6.3 [1.7]	ND[+]	ND[+]
Tetrahymena pyriformis	25.0 [6.8]	25.0 [6.8]	12.5 [5]	6.3 [2.5]

[+]ND = not done.

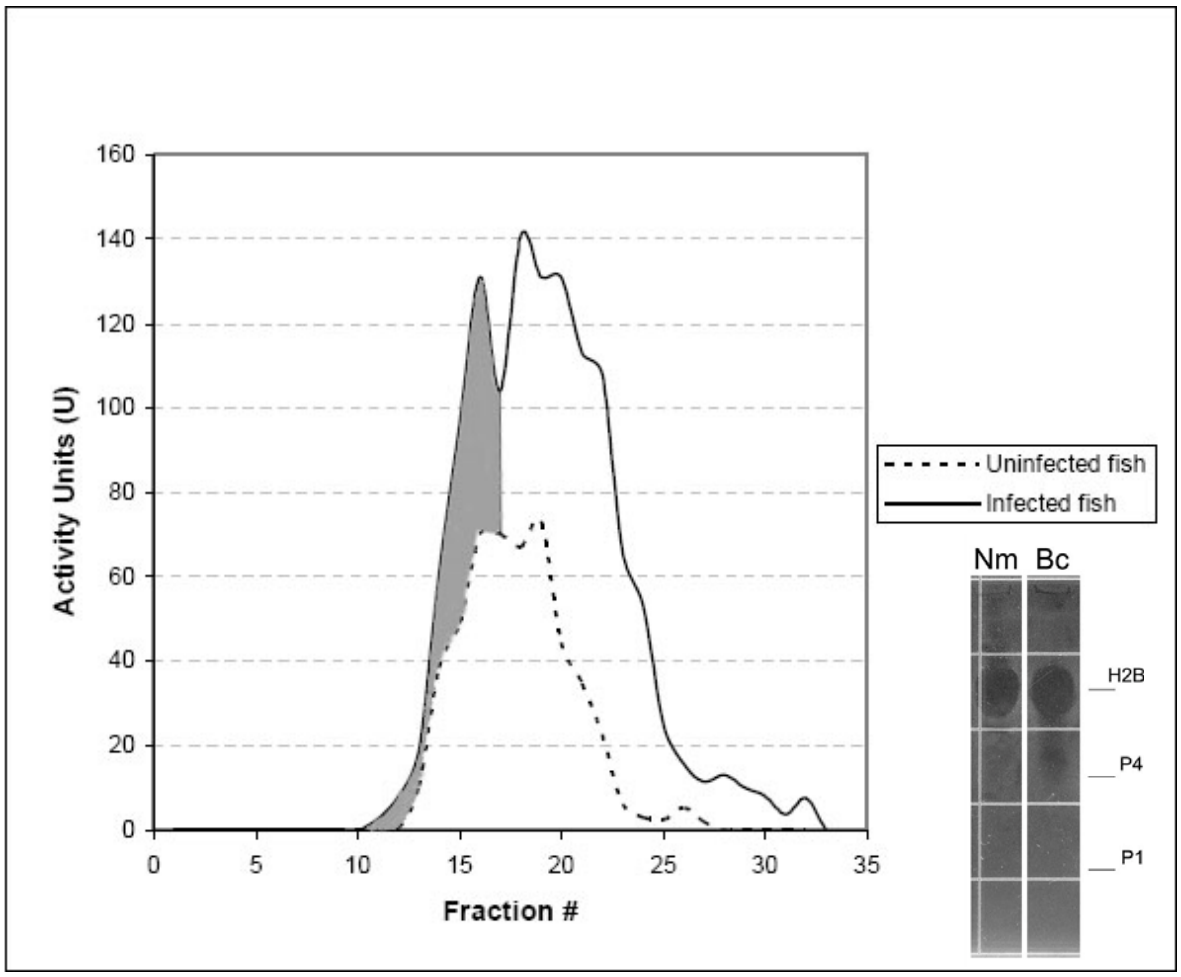


Fig. 1.1. Comparison of activity recovered from CAU-PAGE of pooled channel catfish gill extract from clinically normal fish (dashed line) and fish that had recently recovered from ich infection (solid line). For the ich-exposed fish, a peak is observed at fraction migrating faster than the region having histone-like proteins (fractions 17-24). Fractions from the shaded area (Fxs #11-16) were pooled for subsequent purification via RP-HPLC.

Inset: DAU-PAGE of pooled gill extracts from fish recovering from bacterial infection (Bc) and clinically normal fish (Nm). Note stronger activity in recovering fish extending to the level just above piscidin 4.

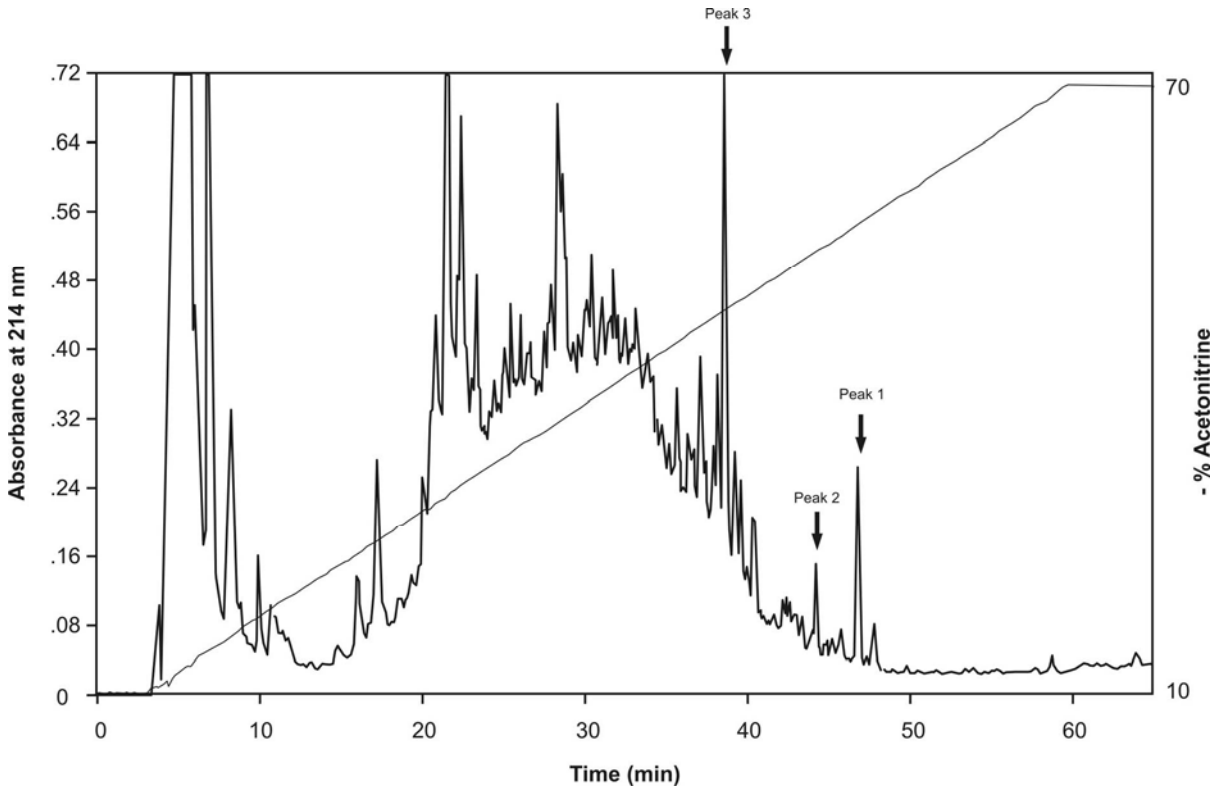


Fig. 1.2 RP-HPLC of CAU-PAGE pool from ich-infected fish showing the three major activity peaks. Of all peaks in the chromatogram, HbβP-1 (Peak 1) had the strongest antibacterial activity, followed by HbβP-2 (Peak 2) and HbβP-3 (Peak 3).

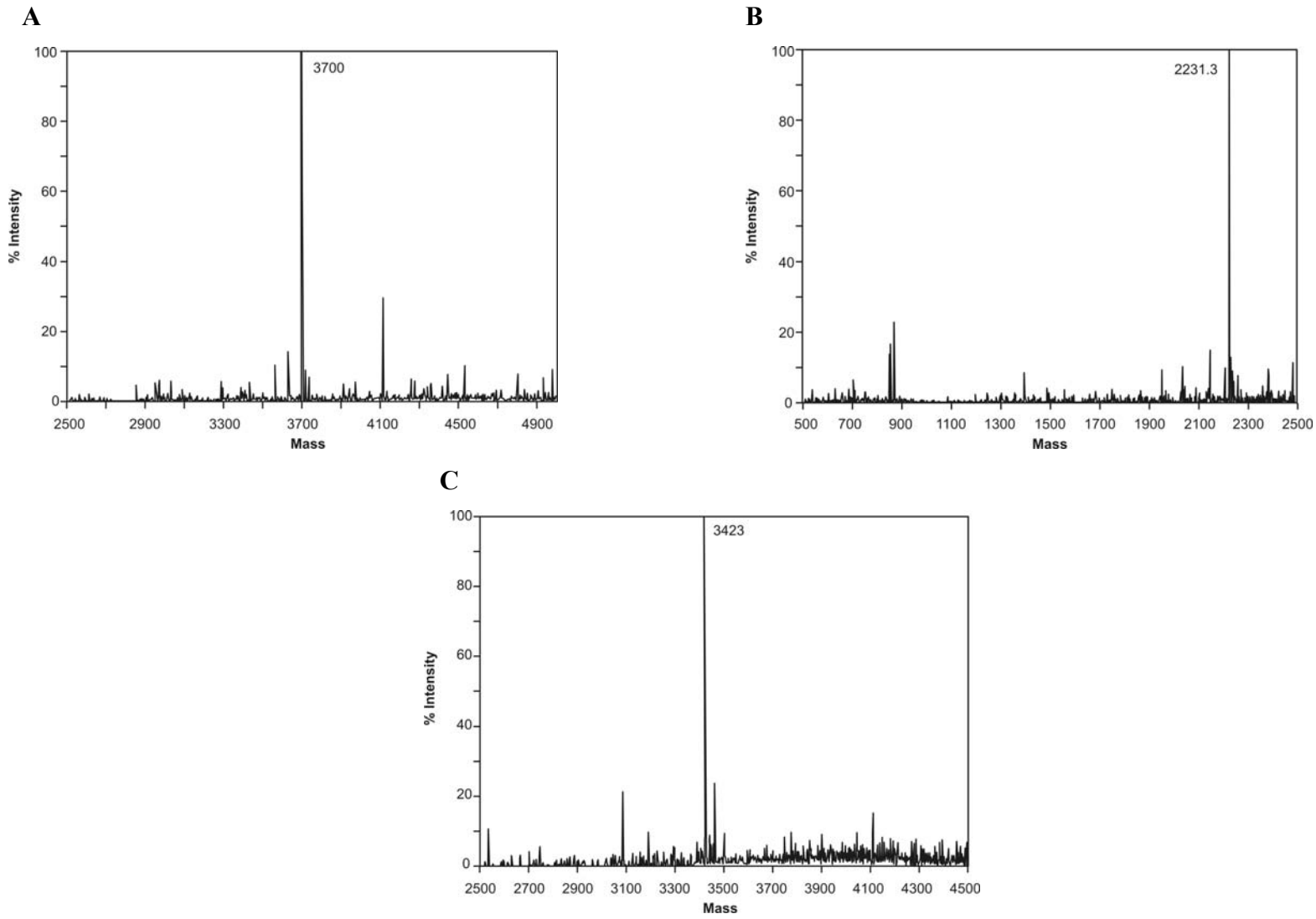


Fig. 1.3 Electrospray ionization mass spectrometry (ESI-MS) of purified HbβP-1 (A), HbβP-2 (B) and HbβP-3 (C) with the transformed spectrum indicating masses of 3700.1 Da, 2231.3 Da and 3423 Da respectively.

M V H W T D A E R H I I A D L W G K I N H D E I G G Q A L A R L L I V Y P W T Q

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40

HbβP-3 (residues 2-9 sequenced; remainder predicted by mass spectroscopy)

R Y F S S F G N L S N A A A I I G N P K V A A H G K V V L G G L T K A V Q N L D

41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80

N I K G I Y T Q L S T L H S E K L H V D P S N F T L L G D T F T V T L A A **K** F G

81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120

Residue 118 is substituted with K in place of N in HbβP-1

P S V F T P E V H E T W Q K F L N V V V A A L G K Q Y H

121 122 123 124 125 126 127 128 129 **130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148**

HbβP-2 is indicated by the residue numbers in bold

Fig. 1.4. The complete amino acid sequence of channel catfish hemoglobin-β as translated from the coding sequence (GenBank, gi:38606321). The Hb-β sequence corresponding to HbβP-3 is residue 2-31 (underlined) while HbβP-1 corresponds to residues 116-148 (underlined). The single amino-acid substitution at position 118 (K for N) is indicated in bold. Residues 130-148 (bold) correspond to HbβP-2, which is homologous to HbβP-1.

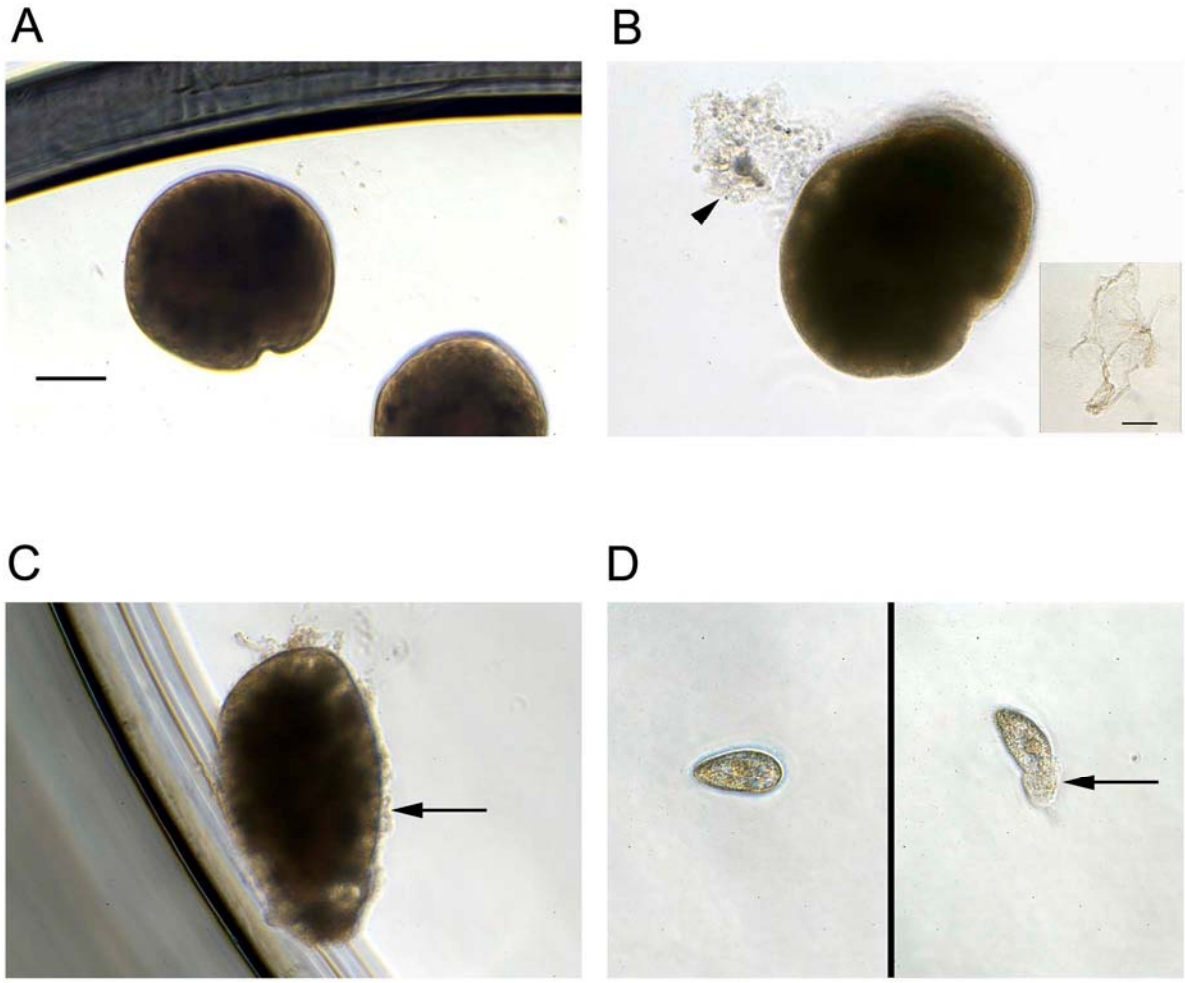
Fig. 1.5. Activity of HbβP-1 against *Ichthyophthirius multifiliis* (ich) and *Tetrahymena pyriformis*.

A) Untreated ich trophont. Notice the homogeneous, dark cytoplasm and the smooth cell membrane. Bar = 100 μm.

B) Ich trophont exposed to 25 μg/ml of HbβP-1 for 1 min. This image is post-excystment since trophonts are too agitated (move too rapidly) to photograph during encystment. Note the parasite has exited the cyst (arrow head and inset). Bar = 100 μm.

C) Ich trophont beginning to lyse after exposure to 25 μg/ml HbβP-1 for 2.5 min. Note membrane blebbing, with the cytoplasm appearing mottled at the cell periphery. Cytoplasm has begun leaking from the damaged cell membrane (arrow). Bar = 100 μm

D) *Tetrahymena*: Untreated (left panel) or exposed to 25 μg/ml of HbβP-1 for 3 min (right panel). The treated parasite is lysing, with cytoplasm leaking from the damaged plasma membrane (arrow). Bar = 50 μm


A
B
C
D

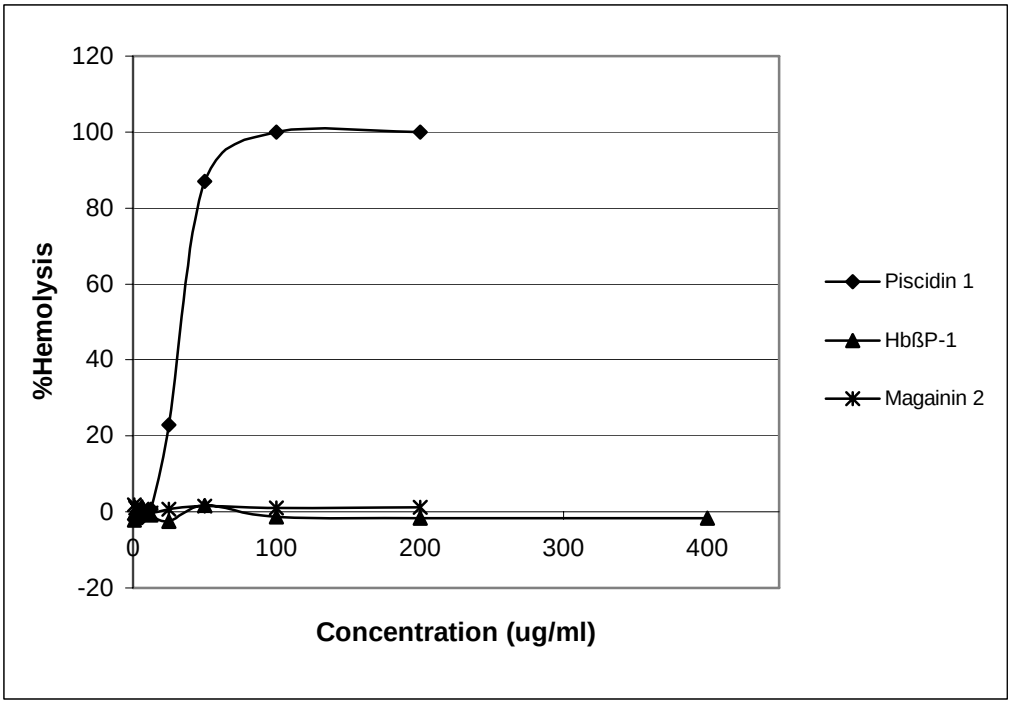
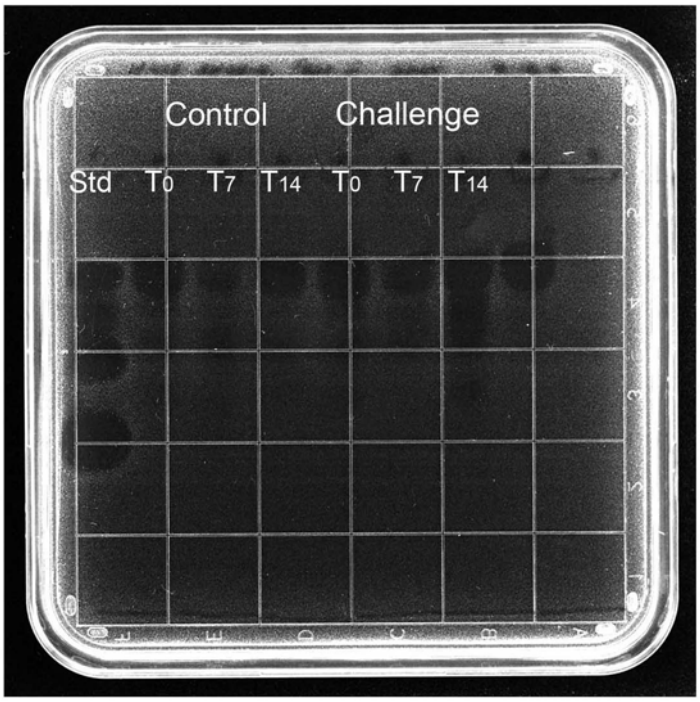


Fig. 1.6. Hemolytic activity of HbβP-1, piscidin 1 and magainin 2.

Fig. 1.7. DAU-PAGE overlay assay ("bug-blot") showing upregulation of antibacterial activity in skin and gill from fish challenged with ich. Pooled skin and gill extracts (three fish per pool) from unchallenged or challenged fish were tested pre-challenge (T_0) and 7 or 14 days post-challenge. Note the greatly increased activity just below the migration zone of histone-like proteins (migrate at level of calf histone H2B). This lower molecular weight zone corresponds to the level of HbβP-1. Replication of this experiment yielded similar results.

Nm – Gill extract pool from two adult clinically normal fish, In – Gill extract pool from the fish recovering from a spontaneous ich infection (chromatogram shown in Fig. 1.1).

Skin


Control
Challenge
Std
T0
T7
T14
T0
T7
T14
H2B
HbβP-1
P4
P1

Gill

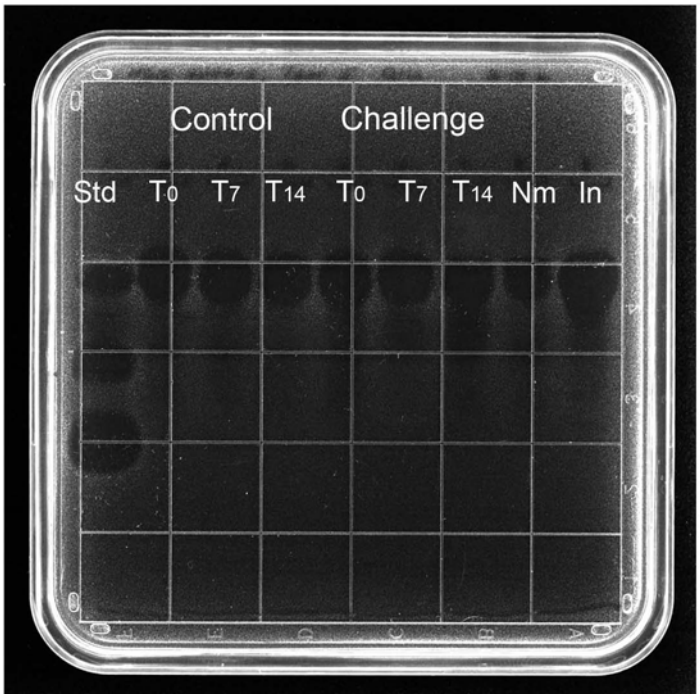

Control
Challenge
Std
T0
T7
T14
T0
T7
T14
Nm
In
H2B
HbβP-1
P4
P1

Fig. 1.8. Immunohistochemistry of channel catfish skin and gill tissue before and after challenge with ich, using anti-HbβP-1 antibody.

A) Skin section from a challenged fish showing strong positive signal in epithelial cells (arrows) in some of the damaged areas probably left behind after exit of parasite. Bar = 200 μm

B) Higher magnification of Fig. 1.8A showing immunopositive epithelial cells. Bar = 50 μm

C) Sham-treated infected skin tissue. Bar = 50 μm

D) Skin from uninfected fish with very weak signal in the epithelium suggesting some sites of constitutive expression. Bar = 200 μm

E) Higher magnification of Fig. 1.2D showing weak signal in epithelium. Immunopositive erythrocytes are present in a blood vessel in the dermis and muscle (arrow). Bar = 50 μm

F) Sham treated uninfected skin tissue. Bar = 50 μm

G) Infected gill tissue with strong signal in the epithelium (arrows) around the parasite. Bar = 25 μm

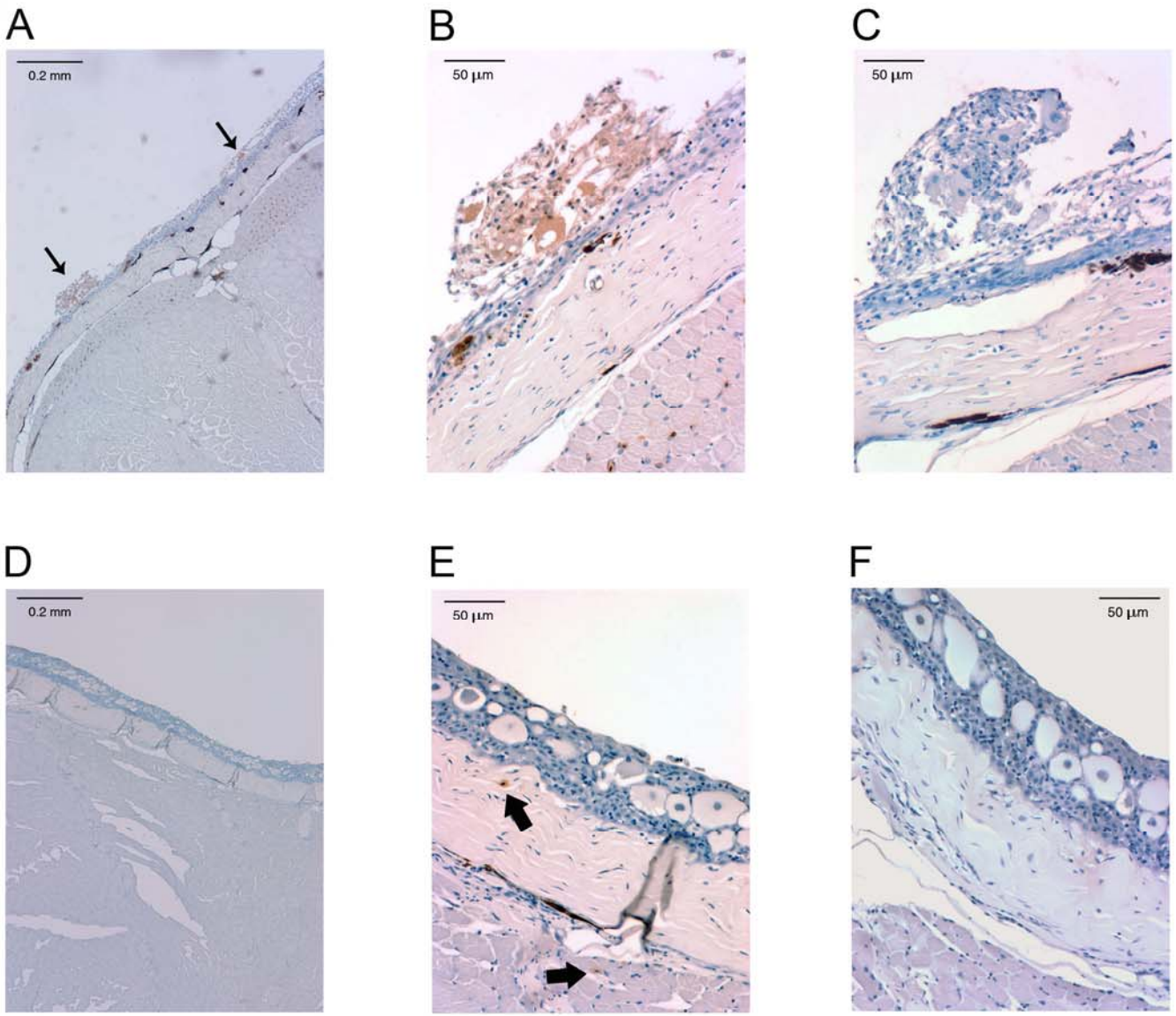
H) Sham-treated infected gill tissue. Bar = 25 μm

I) Immunopositive signal within a parasite (p) within the gill epithelium. No signal is seen in two adjacent parasites. Bar = 50 μm

J) Sham treated parasite. Bar = 50 μm

K) Uninfected gill tissue showing immunopositive erythrocytes (arrows) in secondary lamellae. Bar = 25 μm

L) Sham treated uninfected gill tissue. Bar = 25 μm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0.2 mm
B
50 µm
C
50 µm
D
0.2 mm
E
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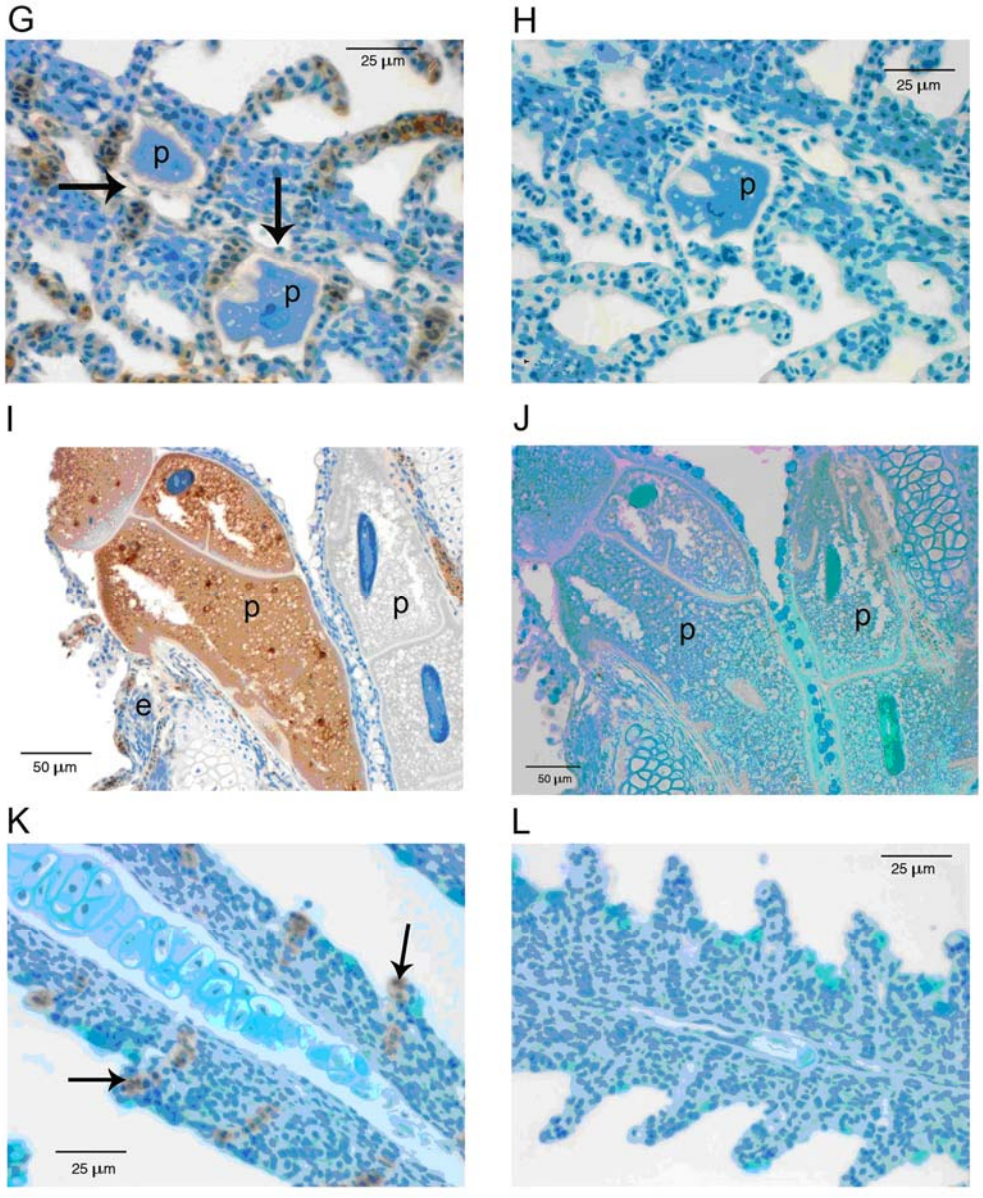
F
50 µm


G
25 µm
p
p
H
25 µm
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I
p
p
e
50 µm
J
p
p
50 µm
K
25 µm
L
25 µm

Fig. 1.9. *In-situ* hybridization of channel catfish skin and gill tissue before and after challenge with ich, using anti-HbβP-1 DIG-labeled probe.

A) Skin of uninfected fish with hybridization signal observed in epithelium. Bar = 50 μm.

B) Sham treated uninfected skin. Bar = 50 μm.

C) Skin of uninfected fish treated with sense probe showing no hybridization. Bar = 50 μm.

D) Skin of infected fish showing stronger hybridization signal in epithelial cells and alarm cells (arrows). Bar = 50 μm.

E) Sham treated infected skin. Bar = 50 μm.

F) A parasite (p) in skin with strong signal in surrounding epithelium and some signal within its cytoplasm. Bar = 50 μm

G) Sham treated section with parasite. Bar = 50 μm.

H) Uninfected gill tissue showing strong hybridization signal in epithelial cells of secondary lamellae. Bar = 50 μm.

I) Sham treated uninfected gill. Bar = 50 μm.

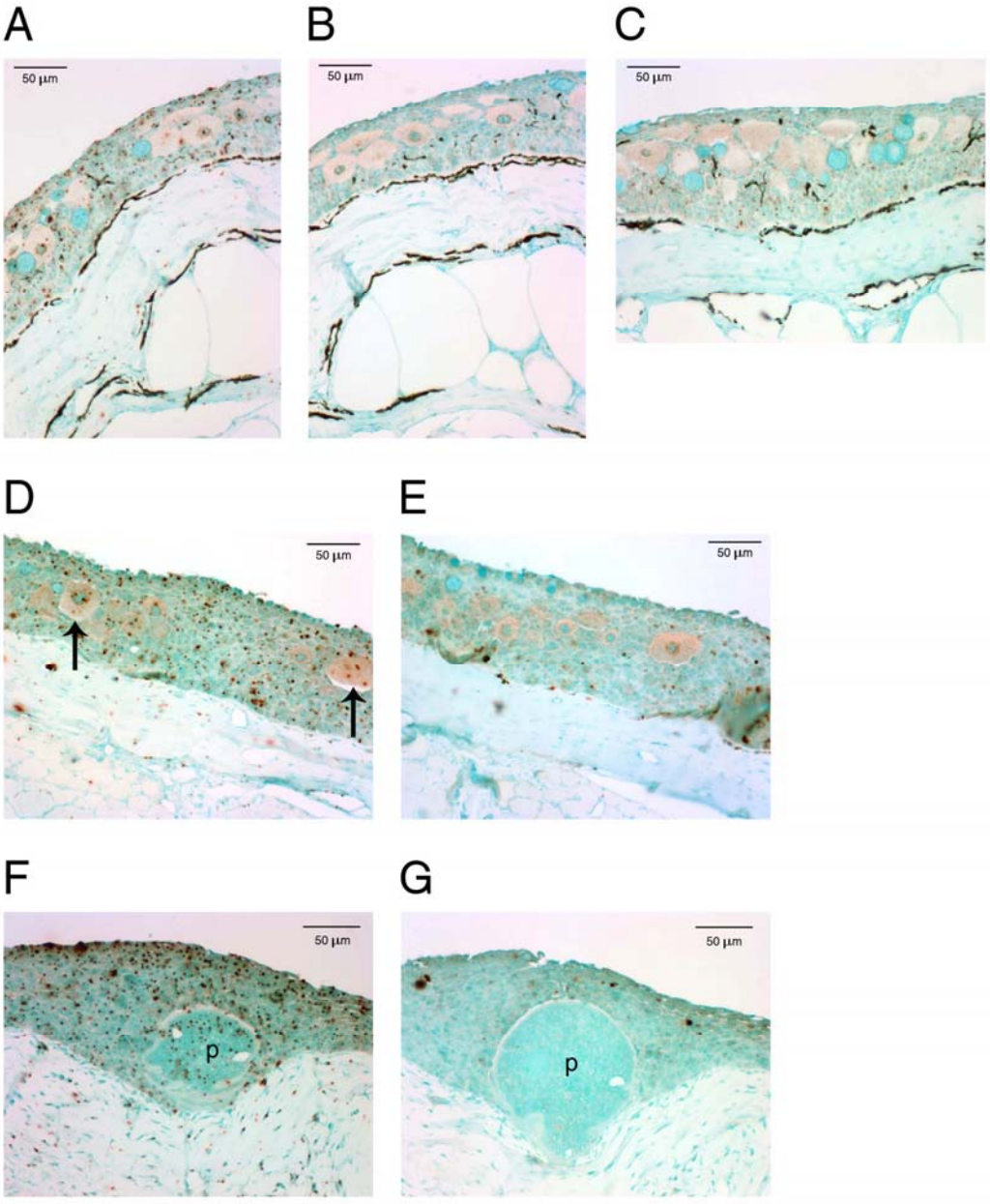
J) Uninfected gill tissue treated with sense probe showing no hybridization. Bar = 50 μm.

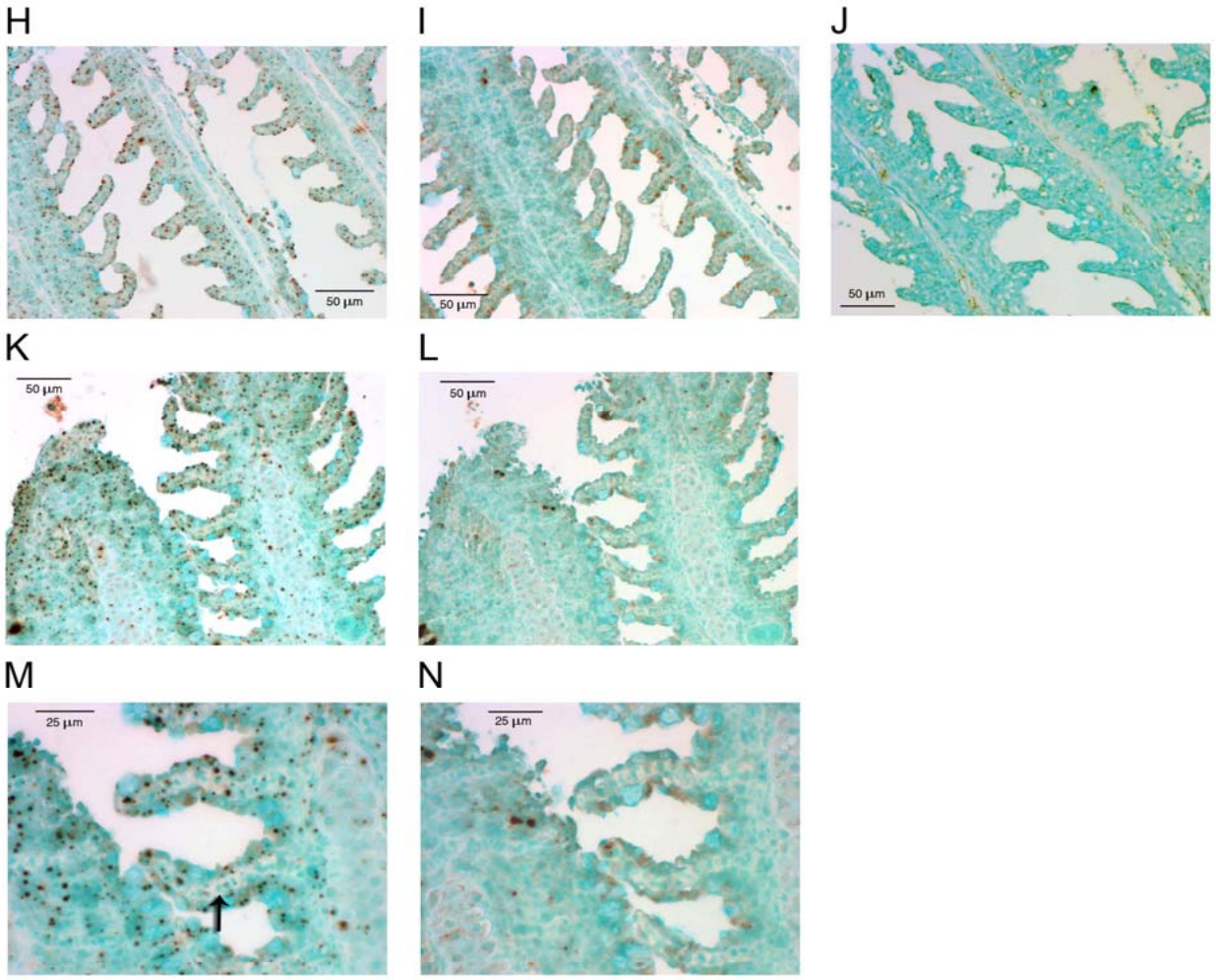
K) Gill from ich infected fish showing much greater hybridization signal in epithelium compared to uninfected gill. 50 μm.

L) Sham treated gill from infected fish. Bar = 50 μm.

M) Strong signal in epithelial cells but not in erythrocytes (arrows) in the middle of the secondary lamellae of infected fish. Bar = 25 μm.

N) Sham treated gill at high magnification (40X). Bar = 25 μm.


A
B
C
D
E
F
G
50 µm
p


H
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K
50 µm
L
50 µm
M
25 µm
N
25 µm

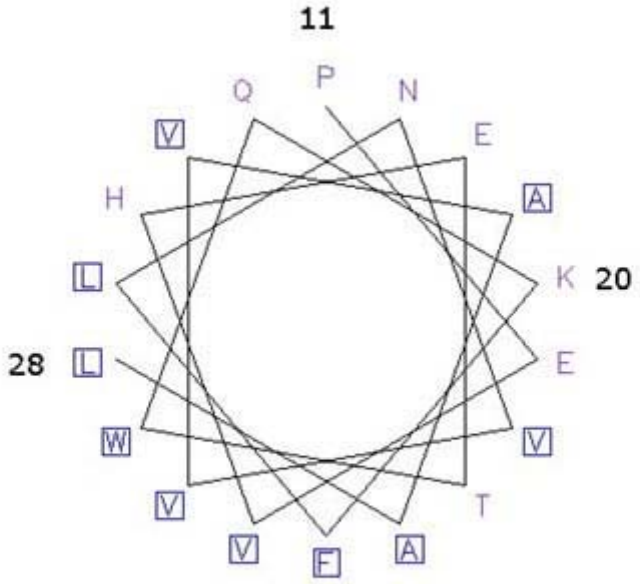

Fig. 1.10. The predicted alpha-helical structure of the helical region (residues 11-28) of the 33 aa HbβP-1 peptide drawn using PEPWHEEL tool (EMBOSS GUI, http://www.hku.hk/EMBOSS/) showing a putative amphipathic property to the helical motif of the peptide. Hydrophobic residues are bordered by a square; the hydrophilic residues are not boxed.

Chapter 2

Hemoglobin: an antiparasitic defense in vertebrates?

(Prepared as manuscript for publication: Ullal, A. J. and Noga, E. J.)

I. ANTIMICROBIAL PEPTIDES (AMPs) AS ANTIPARASITIC DEFENSES

The innate immune system is typically considered the first line of host defense. Both its cellular and non-cellular components recognize a pathogen's membrane components such as lipopolysaccharide (LPS) in gram-negative bacteria, teichoic acid in gram-positive bacteria and mannans, conserved components of yeast cell walls, mounting an immediate defense that leads to recruitment of adaptive, specific immunity in higher vertebrates. Thus, innate immunity facilitates the selection of appropriate antigens and the strategies to eliminate them thereby playing an instructive role in the acquired immune response (Fearon and Locksley 1996). The existence of innate immunity across the animal, plant and even prokaryotic taxa is indication of its evolutionary advantage to host defense. Phagocytes, granulocytes, NK cells, cytokines (interleukins, interferon), C-reactive protein and even enzymes like lysozyme play distinct roles in the innate immune system (Janeway et al 1997).

Recently, increasing focus has shifted to antimicrobial peptides (AMPs); to date, over 880 have been described from animals (http://www.bbcm.units.it/~tossi/pag5.htm)! AMPs are mostly 10-50 residues, typically cationic, and possess broad-spectrum activity that can be directed at bacteria, fungi, eukaryotic parasites, enveloped viruses or even cancer cells (Hancock and Scott 2000). Further evidence suggests that these peptides act synergistically with other components of the innate immune system to mount a rapid and debilitating attack on invading pathogens (Hancock and Scott 2000). As would be expected, a primary location of the AMP defense is in the epithelial surfaces that are in closest contact with the environment (skin, gut and respiratory). But they have also been isolated from other tissues

including bone and testes (Agerberth et al 1995, Warnke et al 2006). They are also present in neutrophils and macrophages, being responsible for a major nonoxidative bactericidal mechanism in these phagocytes (Hancock and Scott 2000). Many AMPs can be induced by pathogenic challenge (Cociancich et al 1994, Hiratsuka et al 1998, Shike et al 2002), while others are constitutively expressed (Silphaduang and Noga 2001).

AMPs have been divided into groups based on shared characteristics, including secondary structure (α-helical or β-sheet), abundance of certain amino acids (e.g., histidine, proline, glycine or cysteine), presence of disulfide bridges (in those that are rich in cysteines), or presence of modified amino acids (Bulet et al 1999, Epand and Vogel 1999, Reddy et al 2004, Wang and Wang 2004).

AMPs exhibit considerable structural diversity even within classes; the cysteine-rich defensins are a well-known example. They are the only class of AMPs that are present in plants, invertebrates and vertebrates (Thomma et al 2002). All plant defensins identified so far have a triple-stranded β-sheet structure with a α-helix in parallel and eight cysteines forming four structure-stabilizing disulfide bridges. Most insect defensins have a double stranded β-sheet with an α-helix and are stabilized by three disulfide bridges between six cysteines. However, drosomycin (four disulfide bridges) and heliomicin (three disulfide bridges) combine a triple-stranded β-sheet with the α-helix. Arthropod defensins have an α-helix and two antiparallel β-sheets with three or four disulfide bridges. Mammalian defensins are further divided into three families (α-, β- and θ-defensins). The α-defensins have a β-sheet structure stabilized by three disulfide bonds but do not have an α-helix motif while θ-defensins are cyclic and believed to be ligated α-defensin dimers (Tang et al 1991). The mammalian β-defensins combine an α-helix with a triple-stranded anti-parallel β-sheet. The

size and spatial orientation of the mammalian β-defensin is comparable to that found in plant defensins since the α-helix appears approximately in the same position relative to the β-sheets in the folded conformation (Hwang and Vogel 1998). Hence, it is believed that there is a closer relationship between mammalian β-defensins and plant defensins than between the mammalian α- and β-defensins (see review; Thomma et al 2002). These cysteine-rich peptides have activity against bacteria, fungi and enveloped viruses (Gallo et al 2002; Hoffman et al 1999; Lehrer and Ganz 2002).

Pioneering work on antibacterial peptides resulted from research carried out in insects mainly because their innate immune system is the only mode of host defense in this class of the animal kingdom that contains by far the most species. This work highlighted the economical use of DNA and a much faster reaction time upon infection resulting in a significant evolutionary advantage for this system of host defense. It also identified numerous AMPs like mellitin and cecropins (amphipathic, α-helical), proline-rich drosocin, apidaecin and abaecin, along with many others (Hultmark 1994, Barra et a. 1998, Otvos 2000).

Antimicrobial activity of AMPs has been mostly assayed using bacteria and fungi, largely due to the ease of testing activity against these pathogens as well as because of their importance to human health. In addition, early studies of AMPs found them generally ineffective against protozoan cell membranes (Rivas and Andreu 2003). Also, the antibacterial, antifungal (and antiviral) properties of AMPs have been of greater economic significance to the developed nations. Antiparasitic studies of AMPs has been relatively limited and focused mainly on *Plasmodium* and *Leishmania* spp., the causative agents of malaria and leishmaniasis, respectively (Vizioli and Salzet 2002) (Table 2.1).

Linear AMPs like magainins, originally isolated from the skin of the African clawed frog (*Xenopus laevis*) (Zasloff 1987) and cecropins (the first inducible AMP to be isolated) from the giant silk moth (*Hyalophora cecropia*)(Steiner et al 1981), are active against *Plasmodium falciparum, P. cynomolgi* and *P. knowlesi* oocysts in several mosquito species (*Anopheles gambiae, A. freeborni* and *A. dirus*)(Gwadz et al 1989). Magainins are also lethal to the ciliate *Paramecium caudatum* (Zasloff 1987, 1988). Both peptides are not hemolytic to sheep and human red cells at protozoacidal concentrations (Gwadz et al 1989, Boman et al 1989). Two insect defensins, from *Aeschna cyanea* (dragon fly)(Bulet et al 1992) and *Phormia terranovae* (flesh fly)(Lambert et al 1989), caused abnormal development of *Plasmodium gallinaceum* oocysts in mosquitoes (*Aedes aegypti*) when injected more than 4 days after a blood meal containing the parasite, and were cidal to isolated sporozoites in vitro; there was no detectable toxicity to mosquitoes at antiparasitic concentrations (Shahabuddin et al 1998). Temporin A and B, 13-residue linear AMPs with a single positively charged residue, isolated from the European red frog (*Rana temporaria*)(Simmaco et al 1996) are cidal to *Leishmania donovani* and *L. pifanoi* promastigotes and amastigotes (Mangoni et al 2005). Dermaseptin, a 34-residue, cationic, linear peptide, isolated from the skin of the South African arboreal frog, *Phyllomedusa sauvagii* (Mor et al 1991), is cidal to *Leishmania mexicana* (Hernandez et al 1992). Several studies have attempted to optimize antimalarial and trypanolytic activities of native AMPs by designing modified isomers and synthetic hybrids based upon charge distribution, amphipathic and hydrophobic properties of the native peptides. These in vitro studies were aimed at combining strong antiparasitic activities while reducing toxicity to host cells (Boman et al 1989, Rodriguez et al 1995, Jaynes et al 1988) and met with limited success.

Human β-defensins have weak trypanicidal activity, while the various cyclic and linear mammalian cathelicidins (SMAP-29, ovispirin, novispirin, protegrin-1) kill procyclic (insect) forms of *Trypanosoma brucei* in vitro and can decrease trypanosomal parasitemia in mice by killing of blood-stream forms of the parasites (McGwire et al 2003). Silva et al (2000) isolated an 18-residue, cysteine-rich, tachyplesin-like AMP from spider (*Acanthoscurria gomesiana*) hemocytes that was cidal to *Leishmania donovani* promastigotes. A 57-residue, cysteine-rich AMP called gambicin was recently isolated from a mosquito (*Anopheles gambiae*) that was lethal to *P. berghei* ookinetes (Vizioli et al 2001). Stomoxyn, an α-helical, amphipathic peptide from stable flies (*Stomoxys calcitrans*) was strongly lytic to *Trypanosoma brucei rhodesiense* trypomastigotes. Scorpine, an AMP isolated from the venom of the scorpion (*Pandinus imperator*), resembles a cecropin-defensin hybrid and has potent cidal effect on the ookinete and gamete stages of *Plasmodium berghei*.

Although there are some studies describing bacteria-induced upregulation of host AMPs that protect against parasite infection (Lowenberger et al 1996, Lowenberger et al 1999, Hao et al 2001), only three studies have demonstrated upregulation of an AMP after parasite challenge. Fruit flies (*Drosophila melanogaster*) upregulate expression of the AMPs, drosocin, drosomycin and diptericin, in their hemolymph and gut, in response to kinetoplastid flagellate gut parasites (*Crythidia bombi* and *C. fasciculate*) that were fed or injected into the thorax of the flies (Boulanger et al 2001). Mosquitoes (*Anopheles gambiae*) fed on *Plasmodium berghei*-infected mouse blood had increased expression of an arthropod defensin mRNA in the midgut within 24 hr of challenge (Dimopoulos et al 1997). Per os challenge with *Leishmania major* induces increased sand fly (*Phlebotomus duboscqi*)

defensin expression in the midgut during the promastigote development stage (day 4) of parasite infestation of the vector. This defensin had weak antiparasitic activity (IC_{50} of 68-85 μM) against the promastigote forms of *L. major* but had strong bactericidal and fungicidal activity (Boulanger et al 2004).

II. PRODUCTION OF ANTIMICROBIAL PEPTIDES FROM OTHER FUNCTIONAL PROTEINS

While the great majority of AMPs are directly derived from genes that code for them, an increasing number of AMPs have been discovered that are derived from larger proteins with primary functions unrelated to a direct microbicidal activity. Histones are DNA-binding proteins that serve to stabilize the chromatin structure in all nucleated cells. As early as the mid-twentieth century, these nuclear proteins were shown to have antibacterial activity (Hirsch 1958). This discovery was ignored for many years, but recent studies in several lower vertebrates have provided evidence for the role of histone-derived AMPs in host defense. Parasin I, a 19-mer AMP, is formed from cleavage of histone H2A by cathepsin D (Park et al 1998). Cathepsin D is generated from procathepsin D by release of a metalloprotease during epidermal injury of Asian catfish (*Parasilurus asotus*)(Cho et al 2002). Histone H2A is also cleaved by pepsin to form the AMP buforin I in the Asian toad (*Bufo bufo gargarizans*) (Kim et al 2000). Histone-derived peptides from mucus and blood of coho salmon (*Oncorhynchus kisutch*), while having no in vitro antibacterial activity, potentiate the activity of the flounder

pleurocidin as well as chicken lysozyme and extracts from the mucus and serum of coho salmon that contain lysozyme (Patrzykat et al 2001).

Intact histones are also increasingly implicated in host defense of many animals. Murine macrophages express MUMPs (murine macrophage proteins) that are highly homologous to histones H1 and H2 (Hiemstra et al 1993) and function in nonoxidative microbial killing. Pacific white shrimp (*Litopenaeus vannamei*)(Patat et al 2004) express proteins related to histones H2A, H2B, H4 and H3 that have activity against *Micrococcus luteus*. A buforin I homologue synthesized from shrimp histone H2A (2-39) also had strong bactericidal activity against gram-positive and gram-negative bacteria (Patat et al 2004). Histone-like proteins related to histones H2B and H1, isolated from the skin, gill or spleen of sunshine bass (*Morone saxatilis* male x *M. chrysops* female), channel catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss*), show strong antibacterial and antifungal activity, as well as potent antiparasitic activity against the important fish parasite *Amyloodinium ocellatum* (Robinette et al 1998, Noga et al 2001, 2002). Further evidence for the role of histones in host defense is their localization in microsomes of gill mucosa of both primitive and advanced teleost fish (Schuurmans Stekhoven et al 2004). Rose et al (1998) also identified histones in villus epithelial cells of the human gastrointestinal tract and suggest that exfoliated, apoptotic epithelial cells may release these proteins and their fragments into the intestinal lumen to serve as antibiotics. A cytoplasmic pool of histones is also present in mammalian liver cells (Zlatanova et al 1990).

Proteolysis of proteins with a well-known antimicrobial function may also generate AMPs; these AMPs are often more potent than the parent protein. Lactoferrin is an 80 kDa glycoprotein consisting of two homologous, globular lobes comprising its amino- and

carboxy-terminal halves that are separated by a cleft. There is a single iron-binding site in the cleft (Grossman et al 1992). It is found in mammalian milk, saliva, mucosal surfaces and seminal fluid, as well as the granules of polymorphonuclear leukocytes (PMNs). Lactoferrin performs numerous roles (iron-binding and metabolism, cellular adhesion and immune modulation, coagulation, antithrombotic, anti-tumor) including functioning as an antimicrobial (antibacterial, antiviral)(Levay et al 1995). Acid proteolysis of ingested lactoferrin in the stomach generates 25- and 45-mer peptides from its N-terminus (lactoferricins) that have greater antibacterial and antifungal activity than lactoferrin (see review in Farnaud and Evans 2003).

Cathepsin G is a 223-residue, highly cationic, arginine-rich serine protease, found in the azurophilic granules of PMNs that can undergo auto-proteolysis. Peptide fragments of cathepsin G have greater antibacterial activity than the native molecule. One of these peptides was isolated from unstimulated PMNs, strongly suggesting that cathepsin G could be proteolytically processed in vivo to yield AMPs (Shafer et al 2002).

Lysozyme is a 129-residue basic protein, found in PMNs as well as in biological fluids, that can hydrolyse the peptidoglycan layer in the cell wall of gram-positive bacteria; it is usually much less effective against gram-negative bacteria. Four disulfide bridges stabilize the molecule and its enzymatic activity is lost when more than two of these bridges are destroyed. However, both the destabilized molecule (devoid of enzymatic activity), as well as C-terminal fragments have cidal activity against gram-negative bacteria as well (Ibrahim et al 2002, Düring et al 1999).

Aprotinin is a 58-residue, basic, naturally occurring serine protease inhibitor protein found in human blood. Proteolytic cleavage of aprotinin with clostripain resulted in 3 antibacterial peptides (Pellegrini et al 1996) and one antiviral peptide against herpes simplex virus 1 (Pellegrini et al 1994). The most active antibacterial peptide (corresponding to residues 18-39 of aprotinin) had greater activity than its parent molecule (Ibrahim et al 2002).

Hemocyanin (Hcy) is the major oxygen transport protein of mollusks and arthropods. It has highly conserved copper-binding sites that are analogous to the conserved iron-binding sites in hemoglobin (Van Holde and Miller 1995). Hemocyanins are large (400,000 to 9,000,000 daltons), extracellular 6 to 48-mers composed of 70 to 450 kDa subunits, depending on species, circulating in the blood (hemolymph)(Bunn and Forget 1986). Peptides with 95-100% homology to the C-terminus of hemocyanin from shrimp (*Penaeus vannamei* and *Penaeus stylirostris*) had antifungal activity against *Nectria hematococca, Alternaria brassicola, Neurospora crassa, Tricoderma viridae, Fusarium culmorum* and *Fusarium oxysporum*, but no antibacterial activity. These AMPs (PsHCt1, PsHCt2 and PvHCt) were believed to be proteolytic fragments of the C-terminus of hemocyanin. Challenge of shrimp with heat-killed bacteria or fungal spores increased their concentrations, suggesting a role in immune defense. The AMP concentrations peaked at 6 h and remained elevated for 72 h after challenge (Destoumieux-Garzón et al 2001). Astacidin 1, a 16-residue AMP isolated from freshwater crayfish (*Pacifastacus leniusculus*) and active against many gram-positive and gram-negative bacteria, has 100% homology to the C-terminal of crayfish hemocyanin. Astacidin 1 was generated by proteolytic cleavage, with enhanced production in LPS- or glucan-challenged crayfish (Lee et al 2003).

III. HEMOGLOBIN AS A MULTIFUNCTIONAL MOLECULE AND A SOURCE OF ANTIPARASITIC DEFENCE

Since the early 1500's, blood was implicated in the circulation of nutritive components, among other things, which was further supported by the observations of Richard Lower a hundred years later, when he established that pulmonary circulation served the important function of aerating venous blood. About two hundred years later (in 1862), Felix Hoppe established the involvement of the red pigment in blood for binding and transporting oxygen and was also the first to use the term "hemoglobin" (Hb) to describe it. Virtually all of the subsequent research carried out on the protein remained in the realm of its oxygen transporting capabilities (Bunn and Forget 1986).

Hemoglobin forms a tetramer of the type $\alpha_2\beta_2$ with two α-subunits (Hb-α) and two β-subunits (Hb-β) interacting with one another by means of relatively weak, non-covalent bonds. In most vertebrates, the α and β globin gene clusters are found on different chromosomes; this multigene family produces 12 Hb isoforms comprising tetramers of Hb-α or -ζ subunits combining with either Hb-β, -γ, -ε or –δ subunits, some of which may be acetylated or glycated. Normal adult human hemolysate has a mixture of Hb tetramers, with 92% HbA ($\alpha_2\beta_2$), 2.5% HbA_2 ($\alpha_2\delta_2$) and 5% of three glycated Hbs (Bunn and Forget 1986).

In the mid-1950's, Hobson and Hirsch (1958) identified the antibacterial properties of the Hb tetramer and its α- and β-subunits from many animals, including horse, rat, guinea pig, cow, mouse and rabbit, as well as HbA, C, E and H variants from humans (the latter are Hb-β variants). Hobson and Hirsch observed that Hb and its subunits were cidal to

Escherichia coli and *Salmonella* at concentrations ranging from 0.02-0.4 μg/ml. But this did not generate much interest at the time. Beginning in early 1971, a number of studies implicated 3 to 16-residue peptide fragments of Hb as a source of various bioactive compounds, including adrenocorticotropic hormone releasing activity (Schally et al 1971, Chang et al 1980), analgesia (neokyotorphins) (Takagi et al 1979, Fukui et al 1983), opioid function (hemorphins)(Brantl et al 1986, Nyberg et al 1997) or vasoactivity (hemorphins)(Galoyan et al 1997, Ianzer et al 2006). Hb has also been suggested to regulate blood flow due to its ability to bind nitric oxide (Jia et al 1996, Stamler et al 1997). The multifunctionality of Hb as a molecular heat transducer, its ability to interact with erythrocyte membranes and cytosolic constituents and thus influence the cell aging, as well as exhibit monooxygenase-like enzyme activity, have been reviewed (Giardina et al 1995).

Most peptides are derived from the N- or C-terminals of Hb-α or Hb-β and appear to result from nonrandom, stepwise, proteolysis. Hemorphins are 6 to 10-mer peptides possibly produced via hydrolysis by macrophage acidic lysosomal proteases (Dagouassat et al 1996; Fruitier et al 1999). Different suites of bioactive Hb-α and Hb-β fragments are present in certain mammalian tissues, including brain, hypothalamus and bone marrow. Ivanov et al (1997) postulated that these peptides were generated in situ in these specific tissues (i.e., a "tissue-specific peptide pool") as a component of peptidergic regulation of tissue homeostasis. The Hb fragments isolated from these tissues were different for each tissue thus supporting the hypothesis that these bioactive fragments are specific to the tissue of origin and are not produced by erythrocytes (Ivanov et al 2005).

Several studies have recently identified AMPs derived from in vitro (Parish et al 2001) or in vivo (Liepke et al 2003, Mak et al 2004) proteolysis of either Hb-α or Hb-β.

AMPs generated in vitro and in vivo from human Hb-α and Hb-β typically have much stronger antimicrobial activity than the intact tetramer or subunits. Interestingly, some of these AMPs have preferential activity for eukaryotes. A C-terminal, 31-mer fragment corresponding to residues 116-146 of Hb-β had greater activity against bacteria (*Escherichia coli, Staphylococcus aureus*) and a fungus (*Candida albicans*) than the intact subunit or the tetramer (Parish et al 2001). A 35-residue AMP of Hb-β (hHEMβ 111-146), as well as an AMP derived from the C-terminal of Hb-γ (hHEMγ 130-146), have been isolated from human placenta, as well as erythrocytes (Liepke et al 2003). Hb-derived AMPs isolated from human uterine secretions during childbirth (puerperal) (Mak et al 2006) and human menstrual vaginal blood (Mak et al 2004) further supported findings of antimicrobial properties for Hb and suggested a protective role for Hb-derived peptides against uterine infections during physiologically stressful conditions occurring during menstruation and childbirth. A broad range of Hb fragments has been observed in these studies, with some peptides differing by only a single C-terminal amino acid, suggesting a role of carboxypeptidase in the generation of some AMPs. Variation in the cidal activity of these fragments against *E. coli* (Mak et al 2004) and the range of selectivity against gram-negative or gram-positive bacteria and fungi (Parish et al 2001) suggests the potential for very broad-spectrum antimicrobial activity in this peptide pool (Table 2.2). Since most of the research relating to Hb-derived peptide antibiotics is relatively recent, little is known of the possible mechanisms involved in their generation. Some clues may be afforded by the mechanisms deduced or suggested for other antibacterial peptides.

The Hb-β^{minor} gene (a gene in the murine Hb-β cluster) and its protein product, is expressed in LPS and IFN-γ-treated macrophages (Liu et al 1999). It was hypothesized that

this was due to expression of the intact Hb tetramer and thus could be involved in NO consumption, although data was not shown to support this. Only the β but not the α subunit was detected and thus the isolated protein might have been a β_4 tetramer, rather than an $\alpha_2\beta_2$ tetramer, since the latter would only occur if there was transcription of both the Hb-α and Hb-β genes. If true, this could affect NO-scavenging capability. But, this study may suggest an antimicrobial role for Hb-β, as we have seen in channel catfish. The Hb teramer also enhances LPS-induced TNF-α production of mononuclear cells (Jürgens et al 2001). Lipoteichoic acid (LTA), the gram-positive counterpart to bacterial LPS, also activates monocytes and macrophages, and is potentiated by the Hb tetramer (Hasty et al 2006).

Hb-β has been identified in the microsomal fraction of gill tissue (Schuurmans Stekhoven et al 2004). Our recent study (Ullal et al, in preparation) further localizes expression of Hb-β to the skin and gill epithelium of channel catfish and demonstrates an *in vivo* defensive role of a family of Hb-β-like antimicrobial peptides against bacterial and eukaryotic pathogens of fish. One of the AMPs from this family (HbβP-1) demonstrated stage-specific activity against the trophozoite stage of a ciliate parasite of fish. Immunohistochemistry (IHC) identified increased expression of Hb-β in fish experimentally challenged with ich (*Ichthyophthirius multifiliis*). *In situ* hybridization (ISH) further confirmed presence of mRNA transcripts in the epidermis of normal fish and indicated increased expression in experimentally challenged fish.

IV. MIGHT HEMOGLOBIN-DERIVED AMPs PROTECT AGAINST CERTAIN HEMO-PARASITES?

It has been known for some time that persons having certain Hb variants, especially those caused by residue substitutions in Hb-β, including HbS (Sickle Cell variant) (Pasvol et al 1978, Friedman et al 1978), HbC (Agarwal et al 2000, Fairhurst et al 2003) and HbE (Hutagalung et al 1999, Chotivanich et al 2002) are resistant to malaria. This protection was evident in the homozygous as well as heterozygous phenotypes of the Hb variants. Could this protection be due to antiparasitic activity of the peptide fragments generated by endogenous or parasite mediated proteolysis of the Hb variants? The retardation of malarial infection in HbS phenotypes is attributed to the sickling of erythrocytes in low oxygen tension as experienced most likely in capillaries where the infected erythrocytes often sequester. The infected cells are also believed to be eliminated through splenic processing, thus reducing the parasitemia in peripheral blood (Pasvol et al 1978; Friedman et al 1978). However, this oxygen-dependent altered cell morphology is not displayed in HbCC homozygous cells, despite the fact that many of the parasites die in erythrocytes expressing this Hb variant (Fairhurst et al 2003). Similarly, erythrocytes expressing the HbAE heterozygous phenotype are highly resistant to parasite infection compared to the normal (HbAA) phenotype; these cells display relatively normal function despite their microcytosis (Chotivanich et al 2002). Each of these Hb-β variants differs only by a single amino acid substitution at the Glu 6 residue for HbS and HbC and Glu 26 residue for HbE, with a more positively charged Lys residue substituting in both HbC and HbE. The HbβP-1 antiparasitic peptide isolated from

channel catfish by our group (Ullal et al, in preparation) also demonstrates a charge-altering, single Asn 118→ Lys substitution, although this is at the C-terminal end of Hb-β.

The Hb-β variants may be expressed to varying degrees in different vertebrate populations and individuals and could afford them varying degrees of protection from multiple pathogenic infections. An interesting finding in sheep and goats with HbA and HbC phenotypes demonstrated switching from HbA to HbC when made anemic or hypoxic by an intracellular mechanism of Hb-subunit gene regulation (Nienhuis et al 1974). Anemia is a common sign of systemic infections by bacteria or parasites and may consequently result in the Hb shift to a more antimicrobially potent phenotype.

The proteolytic digestion of Hb by some parasites (e.g., *Babesia, Schistosoma, Plasmodium*) adds another dimension to the generation of Hb fragments in vivo and their role under pathogenic conditions. Hb accounts for 95% of erythrocyte cytosolic protein (5 mM concentration) and is readily hydrolyzed in the acidic digestive vacuole of *Plasmodium* by aspartic proteases (plasmepsins I & II) and the cysteine protease falcipain as well as host proteases that may be ingested into the digestive vacuole during feeding (Francis et al 1997). Vacuolar Hb digestion results in peptide fragments which are hydrolyzed to individual amino acids by exopeptidases in the parasite cytoplasm (Kolakovich et al 1997). This allows for generation of multiple Hb fragments of varying lengths, a number of which may possess antimicrobial activity, especially since the parasite utilizes only 16% of the amino acids generated from Hb digestion (Krugliak et al 2002).

Ichthyophthirius multifiliis parasites embedded in gill and skin epithelium of infected blue channel catfish had intact fish erythrocytes or Hb-β in IHC-stained tissue sections,

indicating that Hb-β was possibly being proteolytically processed in the parasites (Ullal et al, in preparation). If true, this Hb-peptide response might define a totally novel defense mechanism; one that is based upon the activation of host defense by the *pathogen* rather than the *host*. That is, this defense may require the direct chemical transformation of compounds (viz. Hb or its subunits) into active defensive chemicals by the *pathogen*.

The midgut of blood-feeding parasites such as ticks is exposed to both pathogenic and non-pathogenic microbes. Fragments from bovine Hb digestion in the midgut of a hard tick, *Boophilus microplus* (Fogaca et al 1999) and rabbit Hb in the soft tick, *Ornithodoros moubata* (Nakajima et al 2003) as well as the American dog tick, *Dermacentor variabilis* (Sonenshine et al 2005) had antibacterial activity that was presumed to function as a defense against bacteria in the midgut. This presents an example of proteolytic modification of host Hb by the mammalian blood parasites to defend against bacterial pathogens in the midgut.

The binding of myoglobins and Hb to NO has been suspected to provide protection to malarial parasites (*Plasmodium spp.*)(Taylor-Robinson 1998) as well as other parasites like *Trypanosom cruzi* (Ascenzi et al 2001) from the parasiticidal effects of NO (Taylor-Robinson 1998) that is produced by macrophages. Proteolytis may not only generate Hb fragments with strong antimicrobial activity but would also eliminate the NO-scavenging ability of Hb that may otherwise protect parasites from macrophage-mediated killing. Hb-β may undergo proteolytic modification by enzymes in a manner similar to that seen for histone H2A since it is localized in identical cell types and is seen to generate proteolytic cleavage fragments with antimicrobial activities. This may be true for Hb-α as well, since non-erythroid expression of both Hb-α and Hb-β has now been demonstrated in human, rat and mouse alveolar epithelial cells (Newton et al 2006).

Synergism of these fragments among themselves and with other humoral components of innate immunity could vastly increase the repertoire of antimicrobial activity. This interaction could potentiate activities of weaker components as well as those that are present in low physiologic concentrations. Synergistic potentiation of activity of mammalian defense peptides, indolicidin, protegrin, LL-37 and bactinecin with each other and with lysozyme supports this assumption (Yan and Hancock 2001). Further evidence for cooperative enhancement was demonstrated with histone-derived peptides from coho salmon (*Oncorhynchus kisutch*), flounder pleurocidin and lysozyme (Patrzykat et al 2001). Most recently, fragments of Hb-α and Hb-β were shown to have antibacterial activities enhanced by two- to five-fold in combination, compared to their individual activity (Mak et al 2004).

CONCLUDING REMARKS

The evidence emerging in recent years is increasingly indicative of a mechanism for the production of strong antimicrobial peptides from larger proteins with well defined primary functions. These AMPs produced by proteolysis could be generated by host or pathogen proteases, each possibly generating separate repertoires of antibacterial activities. The fortuitous abundance of proteins like histones and hemoglobin could account for a large reservoir of substrate that is modifiable by post-translational mechanisms to produce peptide fragments with enhanced antimicrobial properties, including antiparasitic peptides. The wide range of antimicrobial activities of these peptides could further function synergistically with each other, as well as other humoral factors, in eliminating a broad range of infections. This

non-selective generation of proteolytic fragments in the host system could function in a "carpet-bomb" fashion, nonspecifically targeting multiple microbial invaders.

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Table 2.1. Antimicrobial peptides (AMPs) isolated from various invertebrates and vertebrates demonstrating antiparasitic activity.

Peptide	Structure and sequence	Origin	Parasite tested	Other antimicrobial activities	References
Cecropin A Cecropin B	Linear, amphipathic α-helix KWKFLKKIEKVGQNIRDGIIKAGPAVAV VGQATQIAK KWKVFLKKIEKMGRNIRNGIVKAGPAI AVLGEAKAILS	Giant silk moth (*Hyalophora cecropia*)	*Plasmodium* (*P. cynomolgi, P. knowlesi*) *P. falciparum* blood streem forms	Strong: Gram-negative bacteria and fungi Weak: Gram-positive bacteria	Gwadz et al 1989 Boman et al 1989
Magainin 2	Linear, amphipathic α-helix GIGKFLHSAKKFGKAFVGEIMN	African clawed frog (*Xenopus laevis*)	*Plasmodium cynomolgi* and *P. knowlesi*	Gram-negative and positive bacteria. Fungi. Protozoa (*Paramecium caudatum*)	Gwadz et al 1989
Dermaseptin	Linear, cationic α-helix. ALWKTMLKKLGTMALHAGKAALGAA ADTISQGTQ	South African arboreal frog (*Phyllomedusa sauvagii*)	*Leishmania .mexicana*	Bacteria and fungi Strong: gram-positive bacteria and some gram-negative bacteria	Hernandez et al 1992; Mor et al 1991, 1994
Phormicin (defensin)	β-sheet with three disulfide bonds ATCDLLSGTGINHSACAAHCLLRGNRG GYCNGKGVCVCRN	Flesh fly (*Phormia terranovae*)	*Plasmodium gallinaceum* oocysts and sporozoites	Selective against gram-positive bacteria	Shahabuddin et al 1998
Aeschna defensin	β-sheet with three disulfide bonds GFGCPLDQMQCHRHCQTITGRSGGYCS GPLKLTCTCYR	Dragon fly (*Aeschna cyanea*)	*Plasmodium gallinaceum* oocysts and sporozoites	Selective against gram-positive bacteria	Shahabuddin et al 1998
Phlebotomus defensin	β-sheet with three disulfide bonds ATCDLLSAFGVGHAACAAHCIG HGYRGGYCNSKAVCTCRR.	Sand fly (*Phlebotomus duboscqi*) midgut	*Leishmania major* promastigote	Strong: gram-positive bacteria, fungi, yeast. Gram-negative bacteria not tested	Boulanger et al 2004
Temporin A Temporin B	Linear, hydrophobic FLPLIGRVLSGIL LLPIVGNLLKSLL	European red frog (*Rana temporaria*)	*Leishmania* (*L. donovani, L. pifanoi*) promastigote and amastigote forms	Selective against gram-positive bacteria. Fungi and tumor cells	Mangoni et al 2005

Table 2.1 (contd.)

Peptide	Structure and sequence	Origin	Parasite tested	Other antimicrobial activities	References[a]
SMAP-29 (cathelicidin)	Amphipathic helix-loop-hydrophobic C-terminal RGLRRLGRKIAHGVKKYGPTVLRIIRIA	Sheep leukocytes	*Trypanosoma brucei*	Gram-negative and gram-positive.	McGwire et al 2003
Gambicin	β-sheet with 4 disulfide bridges MVFAYAPTCARCKSIGARYCGYGYLNR KGVSCDGQTTINSCED-CKRKFGRCSDGFIT	Mosquito (*Anopheles gambiae*) midgut, fat body, hemocyte	*Plasmodium berghei* ookinetes	Gram-negative bacteria and gram-positive bacteria. Fungi.	Vizioli et al 2001
Stomoxyn	Amphipathic, α-helix. RGFRKHFNKLVKKVKHTISETAHVAKD TAVIAGSGAAVVAAT	Stable fly (*Stomoxys calcitrans*) gut epithelium	*Trypanosoma brucei rhodesiense* trypomastigotes	Gram-positive, gram-negative bacteria. Fungi.	Boulanger et al 2002
Scorpine	Cecropin-defensin hybrid structure GWINEEKIQKKIDERMGNTVLGRMAKA IVHKMAKNEFQCMAN-MDMLGNCEKHCQTSGEKGYCHGTKCK CGTPLSY	Scorpion (*Pandinus imperator*) venom	*Plasmodium berghei* ookinetes and gametes	Gram-positive and gram-negative bacteria	Conde et al 2000
Gomesin	β-sheet with 2 disulfide bridges ZC*RRLC*YKQRC*VTYC*RGR^{+}	Spider (*Acanthoscurria gomesiana*) hemocytes	*Leishmania amazonensis*	Gram-positive and negative bacteria. Fungi, yeast.	Silva et al 2000

$^{+}$C*= Pyridylethylated cysteine. Z = pyroglutamic acid

[a]References cited for antiparasitic activity testing only.

Table 2.2. Antimicrobial peptides and proteins derived from vertebrate hemoglobin generated *in vitro* and *in vivo*.

Subunit	Fragment	Bacterial MIC range (μM)	MIC range (μM) against eukaryotes	Hemolytic/ Cytotoxic activity (μM)	Source	Reference
Hb-α + heme	1-141	0.06 → 0.13 gram –ve → gram +ve	0.95 *C. albicans*	ND	Commercial (human)+	Parish et al (2001)
Hb-α	1-141	0.13 → 0.99 gram –ve → gram +ve	0.46 *C. albicans*	ND		
Hb-α N-terminal	1-76	37-58 gram –ve & gram +ve	58 *C. albicans*	ND	Commercial (human)+	Parish et al (2001)
Hb-α N-terminal	1-32	>30 gram –ve & gram +ve	>30 *C. albicans*	ND		
Hb-α N-terminal	1-40	*E. coli**	ND	ND	Human menstrual blood	Mak et al (2004)
Hb-α N-terminal	1-33	*E. coli**	ND	ND		
Hb-α N-terminal	1-32	*E. coli**	ND	ND		
Hb-α N-terminal	1-31	*E. coli**	ND	ND		
Hb-α N-terminal	1-29	*E. coli**	ND	ND		
Hb-α N-terminal	1-28	*E. coli**	ND	ND		
Hb-α N-terminal	1-27	*E. coli**	ND	ND		
Hb-α N-terminal	1-26	*E. coli**	ND	ND		
Hb-α N-terminal	1-25	*E. coli**	ND	ND		
Hb-α N-terminal	1-20	*E. coli**	ND	ND		
Hb-α mid	17-31	*E. coli**	ND	ND		
Hb-α mid	18-44	*E. coli**	ND	ND		
Hb-α mid	33-76	21.1 – 73.8 gram –ve & gram +ve	14.8 *C. albicans*	ND	Commercial (human)+	Parish et al (2001)
Hb-α mid	35-56	105 → 293 gram –ve → gram +ve	>300 *C. albicans*	ND	Human menstrual blood	Mak et al (2004)

Table 2.2 (contd.)

Subunit	Fragment	Bacterial MIC range (μM)	MIC range (μM) against eukaryotes	Hemolytic/ Cytotoxic activity (μM)	Source	Reference
Hb-α mid	35-58	*E. coli**	ND	ND	Human menstrual blood	Mak et al (2004)
Hb-α mid	35-60	*E. coli**	ND	ND		
Hb-α mid	35-72	*E. coli**	ND	ND		
Hb-α mid	35-77	*E. coli**	ND	ND		
Hb-α mid	35-78	*E. coli**	ND	ND		
Hb-α mid	35-79	*E. coli**	ND	ND		
Hb-α mid	35-80	*E. coli**	ND	ND		
Hb-α mid	35-90	*E. coli**	ND	ND		
Hb-α mid	35-97	*E. coli**	ND	ND		
Hb-α C-terminal	77-141	>14 gram –ve & gram +ve	4.23 *C. albicans*	ND	Commercial (human)+	Parish et al (2001)
Hb-α C-terminal	106-141	*E. coli**	ND	ND	Human menstrual blood	Mak et al (2004)
Hb-α C-terminal	107-141	*E. coli**	ND	ND		
Hb-α C-terminal	107-140	*E. coli**	ND	ND		
Hb-α C-terminal	110-141	*E. coli**	ND	ND		
Hb-α C-terminal	110-131	*E. coli**	ND	ND		
Hb-β + Heme	1-146	0.18 → 0.91 gram –ve → gram +ve	605 *C. albicans*	ND	Commercial (human)+	Parish et al (2001)
Hb-β	1-146	0.25 → 12.6 gram –ve → gram +ve	18.8 *C. albicans*	ND		
Hb-β N-terminal	1-55	2.48 – 16.6 gram –ve & gram +ve	4.14 *C. albicans*	ND		
Hb-β N-terminal	1-30	*E. coli**	ND	ND	Human menstrual blood	Mak et al (2004)
Hb-β mid	16-30	*E. coli**	ND	ND		
Hb-β mid	43-83	*E. coli**	ND	ND		

Table 2.2 (contd.)

Subunit	Fragment	Bacterial MIC range (μM)	MIC range (μM) against eukaryotes	Hemolytic/ Cytotoxic activity (μM)	Source	Reference
Hb-β mid	46-64	*E. coli**	ND	ND	Human menstrual blood	Make et al (2004)
Hb-β mid	56-72	11.5 → 5753 gram –ve → gram +ve	863 *C. albicans*	ND	Commercial (human)+	Parish et al (2001)
Hb-β C-terminal	56-146	0.51 → 6.1 gram –ve → gram +ve	2.5 *C. albicans*	ND		
Hb-β C-terminal	111-146	8 – 51.3 gram –ve & gam +ve	12 *S. cerevisiae*	102	Human placenta	Liepke et al (2003)
Hb-β Cterminal	111-146	*E. coli**	ND	ND	Human menstrual blood	Mak et al (2004)
Hb-β Cterminal	113-146	*E. coli**	ND	ND		
Hb-β C-terminal	115-146	27 → 61 gram –ve → gram +ve	>300 *C. albicans*	ND	Human menstrual blood	Mak et al (2004)
Hb-β C-terminal	116-146	059 → 731 gram –ve → gram +ve	2.9 *C. albicans*	ND	Commercial (human)+	Parish et al (2001)
Hb-γ C-terminal	130-146	52 – 100 gram –ve & gram +ve	52 *S. cerevisiae*	>258	Human placenta	Liepke et al (2003)
Hb-β C-terminal	115-147	3.4 – 13.5 gram –ve only	3.4 – 6.8 *I. multifiliis, T. pyriformis*	>108	Channel catfish skin and gill epithelium.	Ullal et al (in preparation)
Human Hb		0.03 → 309 gram –ve → gram +ve	0.77 *C. albicans*	ND	Commercial (human)	Parish et al (2001)
Alligator Hb		0.3 gram –ve	298 *C. albicans*	ND	Erythrocyte lysate	
Horse Hb		0.03 → 309 gram –ve → gram +ve	3.86 *C. albicans*	ND	Commercial (equine)	
Snake Hb		0.15 → 303 gram –ve → gram +ve	1.52 *C. albicans*	ND	Commercial (snake)	

*MIC not calculated. ND = Not Done.

Hemolytic activity is defined as at least 10% observed hemolysis.

+Fragments generated *in vitro* by CNBr digestion.

Chapter 3

Antiparasitic activity of HbβP-1, a member of the β-hemoglobin peptide family.

(Prepared as manuscript for publication: Ullal, A. J. and Noga, E. J.)

ABSTRACT

A family of antimicrobial peptides (AMPs) derived from β-subunit of hemoglobin was recently isolated from channel catfish (*Ictalurus punctatus*) that were infected with the important freshwater fish parasite *Ichthyophthirius multifiliis* (ich), the causative agent of ichthyopthiriosis. We previously discovered that one of these AMPs, HbβP-1, had strong cidal activity against ich as well as another ectoparasite, *Tetrahymena pyriformis*. HbβP-1 toxicity was specific, primarily affecting the trophozoite (trophont) stage of ich. Here we show that HbβP-1 acted more rapidly to kill smaller (presumably less mature) trophonts of ich, taking almost twice as long to kill the larger trophonts ($p < 0.0001$). It also acted more rapidly than an unrelated AMP, piscidin 1, which is hemolytic and is also lethal to ich trophonts. HbβP-1 was also potently and selectively lethal to the trophont stage of the dinoflagellate ectoparasite, *Amyloodinium ocellatum,* one of the most important pathogens of warmwater marine fish. HbβP-1 had no visible effect on the fish gill cell line feeder layer (G1B cells) used to propagate *Amyloodinium*, further suggesting a highly selective action. These findings suggest that HbβP-1 or related AMPs might function in protecting marine as well as freshwater fish and that HbβP-1 has highly selective activity against specific life stages of important fish ectoparasites.

INTRODUCTION

Protozoan ectoparasites, affecting the skin and gills, cause serious economic losses in cultured fish. Among the most important are the ciliate, *Ichthyophthirius multifiliis*, that causes ichthyophthiriosis (freshwater ich) and the dinoflagellate, *Amyloodinium ocellatum*, that causes amyloodiniosis (marine velvet disease) (Dickerson and Dawe 1995, Noga and Levy 2006). Both parasites have a feeding, trophozoite stage (trophont) in the host epithelium that detaches from the host to form the reproductive, dividing stage (tomont), which produces the invasive, free-swimming stage (theront or dinospore). They are among the most dangerous parasites affecting fish due to their highly invasive nature and ability to penetrate the host epithelium (Woo 1995).

The innate immune system in invertebrates and lower vertebrates serves a crucial role in defending against microbes and parasites (Magnadóttir 2006). Antimicrobial peptides (AMPs) have come to be recognized as important components of this defense (see recent reviews by Fearon and Locksley 1996, Boman 1995, Hancock and Lehrer 1998, and Zasloff 2002), protecting the host in a rapid broad-spectrum manner. To date, there have been over 800 different antimicrobial peptides isolated from eukaryotes (http://www.bbcm.units.it/~tossi/pag5.htm); most have 10 – 50 amino acid residues and are classified based on shared structural characteristics and amino acid composition (Bulet et al 1999, Reddy et al 2004, Wang and Wang 2004). AMPs may be constitutively expressed (Silphaduang and Noga 2001) or induced by pathogenic challenge (Cociancich et al 1994, Hiratsuka et al 1998, Shike et al 2002).

Piscidins, initially isolated from hybrid striped bass (*Morone saxatilis* x *M. chrysops*) and which appear to be present in many teleost fish (Silphaduang et al, In Press), are active against several fish parasites (Noga et al 2001, 2002; Ullal et al, In Preparation), suggesting an antiparasitic role for AMPs of fish. We have recently isolated a family of peptides derived from the β-chain of hemoglobin (Hb-β), one of the two major subunits of this respiratory protein. These AMPs were upregulated in channel catfish (*Ictalurus punctatus*) challenged with *I. multifiliis*. HbβP-1, a member of this family, had relatively weak antibacterial activity but pronounced antiparasitic activity against two ectoparasites, *I. multifiliis* and *T. pyriformis*, with stage-specific activity against the trophozoite (trophont) stage of ich (Ullal et al, In preparation). In these studies we further explored the scope of this activity in comparison to piscidin 1, a potent lytic peptide of comparable antiparasitic activity that is also strongly hemolytic, as well as examined the effect of parasite size (presumably maturity) on antiparasitic activity of HbβP-1.

MATERIALS AND METHODS

Synthesis of HbβP-1

HbβP-1, previously isolated from channel catfish gill (Ullal et al, In preparation), was synthesized via Fmoc chemistry on a Rainin Symphony instrument that provides on-instrument cleavage of the peptide from the resin. After synthesis, the peptide was purified via analytical reverse phase HPLC using a YMC C-18 column (4 mm x 50 mm, 3 micron particle size, 120 angstrom pore size support) using an acetonitrile gradient that was eluted at 1 ml/min where buffer A was 0.05% TFA in water and buffer B was 80% acetonitrile in 0.05% TFA in water. The peptide was detected by its absorbance at 210 nm. Mass spectrometry of an aliquot of purified peptide was carried out on a Micromass TofSpec SE mass spectrometer that was operated in positive ion mode and that was equipped with a nitrogen laser (337 nm), a reflectron, delayed extraction and a post acceleration detector. The purified peptide was lyophilized from 0.05% TFA/acetonitrile solution and stored desiccated under argon gas until reconstitution in solvent. The reconstituted synthetic HbβP-1 peptide was used for all antiparasitic and antibacterial activity tests.

Lethality to *Ichthyophthirius multifiliis*

Ich was propagated on channel catfish fingerlings by adding 3-4 naïve fish every 10-14 days, to a 10 l aquarium having infected fish (Dickerson et al 1985). About 40-50 trophonts were scraped off the skin of infected fish and transferred to 2 ml of filter sterilized

aquarium water in a polystyrene Petri dish. Trophonts were observed under an inverted phase contrast microscope (Nikon Diaphot, Tokyo, Japan) and trophonts were gently pipetted into test wells containing the test peptide dilutions. Trophonts ranging from 150 to 550 μm were selected for the assay. Trophonts were accurately measured once they had been added to the test wells in the microtiter plates.

Test wells of a 96-well flat-bottom polystyrene microtiter plate (Costar, #3208) were loaded with 80 μl of filter-sterilized aquarium water and 10 μl of each peptide dilution. Serial dilutions of piscidin 1 and HbβP-1, suspended in 0.01% Acetic acid (HAc), were prepared in 0.2% BSA in 0.01% HAc at ten times the desired test concentrations and loaded in duplicate wells with the last column of wells having the diluent (0.2% BSA in 0.01% HAc) as a negative control. Three trophonts in 10 μl of aquarium water were added to each test well (duplicate peptide dilutions) to make the final volume to 100 μl. Observations were made every minute for the first 15 min, every 10 min for the next 30 min and then every 15 min for the following 75 min. Test plates were observed every hour for the following 4 hr and then every 24 hr to check for any changes in morphology or development of surviving parasites.

The effect of trophont size on susceptibility to HbβP-1 was also tested. Concentrations of 12.5 and 25 μg/ml were tested against small (180–260 μm) or large (400-600 μm) trophonts that were gently pipetted into triplicate wells (3 trophonts per well, triplicate wells per treatment). Observations were made as described above.

Trophonts were observed for hyperactivity (sudden, rapid and random movement), “encystment” (secretion of a mucoid capsule [“cyst”]), excystment (emergence of the parasite from the cyst), decrease in ciliary movement and finally, death (defined as cessation of all ciliary movement; which was always accompanied by lysis).

Lethality to *Amyloodinium ocellatum*

The effect of HbβP-1 on *A. ocellatum* was examined as per the standard in vitro infectivity assay (Noga 1989). This assay determines the ability of dinospores to infect the G1B monolayer and differentiate into trophonts. Parasites that were isolated from striped bass (*Morone saxatilis*) farmed in the Gulf of Eilat, Israel (Red Sea isolate) were propagated on a gill cell line (G1B cells, ATCC #CRL 2536, American Type Culture Collection, Manassas, VA) adapted to an artificial seawater solution (IO2/HBSS). For experiments, G1B monolayers were gently trypsinized, resuspended in medium (modified Ham's F-12), seeded into 96-well tissue culture plates and allowed to grow to confluency. The monolayers were then adapted to IO2/HBSS. An asynchronous parasite suspension containing dinospores and tomonts was produced by standard procedures and 50 µl was inoculated into replicate wells with various antibiotic concentrations. The number of trophonts and tomonts that formed (i.e., differentiated from dinospores) in each well during incubation at 25°C were counted under phase contrast microscopy after 24, 48, 72 and 96 hr, as described previously (Noga et al 2001). The time to complete destruction of the G1B monolayer was also recorded for each treatment every 24 hr for 10 days.

Effect of HbβP-1 on dinospores was examined by adding 50 µl of a dinospore suspension (prepared as described above) into duplicate wells of a 96-well microtiter plate containing serial dilutions of HbβP-1 or diluent (sham treated), without G1B cells. Some tomonts were also present in the suspension. Dinospores were observed for abnormal motility or death (defined as complete immobilization). Tomonts were observed for arrested

division and death (defined as failure to divide). Observations were made every hour for the first 6 hr, and then every 24 hr for 3 days.

Toxicity to G1B Gill Cells

The G1B cells were seeded into a 96-well microtiter plate and allowed to grow to confluence in modified Ham's F-12 medium as described above. Ten µl of serial dilutions of HbβP-1, prepared at ten times the final desired concentrations, were added to duplicate test wells containing 90 µl of fresh medium. Appearance of the monolayers in test wells was observed every 12 hr over 10 days and compared to duplicate wells having medium alone or diluent alone.

Statistical Analyses

The activity of two HbβP-1 concentrations against two sizes of *I. multifiliis* trophonts was analyzed by Tukey's multiple pair-wise comparison method (α=0.01). HbβP-1 and piscidin 1 activities against *I. multifiliis* trophonts were also compared using the same method. Trophont sizes used to test each peptide were also compared by Student's t-test. All statistical analyses were carried out using PROC GLM in SAS version 9.1 (SAS institute, Cary, NC).

RESULTS

Previous results showed that HbβP-1 was highly selective for the *I. multifiliis* trophont, killing at least some trophonts at as low as 3.4 μM, while showing no lethality against the tomont or theront stages at 54 uM (Ullal et al, In preparation). Because of this stage-selective activity, we tested HbβP-1 against two different trophont sizes; the larger trophonts being presumably more mature, while the smaller parasites are presumably more recently differentiated from the infective theront stage. At both HbβP-1 concentrations (3.4 or 6.8 μM) small trophonts were killed significantly faster than large trophonts, large trophonts took almost twice as long to die ($p < 0.0001$, Tukey's pair-wise test)(Table 3.1). The larger trophonts also underwent "encystment" (secretion of mucilaginous cyst), followed by excystment (agitated exit from the cyst) before ciliary motion began to stop. Encystment/excystment was not observed in the smaller trophonts, which only stopped ciliary activity before lysis.

When HbβP-1 was compared with piscidin 1, HbβP-1 was always more rapidly lethal than piscidin 1 at a similar concentration ($p<0.0001$)(Table 3.2). Piscidin 1 took approximately twice as long as HbβP-1 to kill trophonts at all equivalent concentrations.

Exposure of trophonts to HbβP-1 resulted in immediate (within 5 sec) agitation, hypermotility and increased ciliary motion at all concentrations. Ciliary movement ceased discontiguously. Thus, certain portions of the plasma membrane completely ceased ciliary movement and started to leak cytoplasm at the same time as other portions maintained normal ciliary activity. This degree of agitation was absent with piscidin 1 exposure, which instead lead to a gradual decline of ciliary motion evenly across the entire trophont. Blebbing

of the trophont's plasma membrane and leakage of cytoplasm from damaged regions also appeared more severe with HbβP-1 than with piscidin 1 treatment (Fig. 3.1A, 3.1B, 3.1D). Treatment with low to moderate concentrations of HbβP-1 (1.7 - 13.5 μM; 6.2 - 50 μg/ml) almost always lead to the secretion of a mucilaginous cyst (Fig. 3.1C) followed soon after by excystment and subsequent cell death. This response was not observed at high concentrations (27 - 54 μM) of HbβP-1 or with piscidin 1 at any concentration.

Infectivity was defined as the ability of the dinospores to attach to the G1B monolayer and progress to form the trophont (which typically occurs within 20 min in this culture system, Noga 1987). When tested against *Amyloodinium,* HbβP-1 did not prevent the attachment and differentiation of the dinospores into trophonts at any concentration, as indicated by no effect on infectivity at 48 hr (p = not yet calculated)(Fig 2). However, at 96 hr, exposure to 100 or 200 μg/ml (27 or 54 μM) HbβP-1 completely prevented differentiation of the trophonts into tomonts. Rarely, some trophonts differentiated into tomonts, which began to die at 72 hr and all died within 96 hr. Sham-treated (diluent only) and positive growth control (only artificial seawater) wells had tomonts and some dinospores after 48 hr, with increasing numbers at 96 hr, and rapid destruction of the entire G1B monolayer at 120 hr. HbβP-1 at 13.5 μM (50 μg/ml) reduced infectivity by 30%, with the G1B monolayer surviving up to 48 h longer (168 hr since seeding) than the sham-treated control wells. Lower HbβP-1 concentrations did not affect infectivity (Fig. 3.2) nor had any other apparent effects on parasite growth or survival.

HbβP-1 did not have any visible effect on dinospores or tomonts at any concentration when tested in the absence of the G1B monolayer. Treated dinospores retained motility to the same extent observed in the control wells. Tomonts in both control and test wells started to

form dinospores within 24 hr and, as expected, all dinospores died within 96 hr. G1B cells treated with HbβP-1 in modified Ham's F-12 medium had no visible cytotoxicity at any concentration after 240 hr exposure.

DISCUSSION

Epithelial surfaces of fish, like skin and gill, are living tissues that have a wide variety of host defenses to combat pathogens. A family of AMPs derived from Hb-β is present in the skin and gill epithelium of channel catfish (Ullal et al, In preparation), which are the target tissues for ich, *Tetrahymena* and *Amyloodinium*. Our present study further substantiates the stage-specific activity of HbβP-1 against ich trophonts, the stage that is in most intimate contact with the fish and consequently most damaging to the host. We previously showed that HbβP-1 was only toxic to the ich trophont and affected neither the free-swimming, invasive theront nor the encysted, dividing tomont (Ullal et al, In preparation).

Stage-specific activity of insect defensins on the late oocyst and sporozoite stages of *Plasmodium gallinaceum* found in mosquito mid-gut suggest a possible relationship between the changes in lipid composition of the parasite membrane during different developmental stages and the activity of defensins present in the infected host tissue (Shahabuddin et al 1998). HbβP-1 activity may be similarly affected by changes in membrane composition in different developmental stages of ich. Stage-specific AMP activity has also been demonstrated in vitro for magainin, cecropin and synthetic analogs of cecropin against *P. falciparum* and *Trypanosoma cruzi* (Jaynes et al 1988, Gwadz et al 1989).

The smaller (presumably less mature) trophonts lysed more rapidly than the larger trophonts. This might be due to a physical difference in the shape or composition of the plasma membrane of the smaller trophonts that might be more readily damaged as compared to larger trophonts. A likely advantage of this phenomenon to the host could be in

minimizing epithelial damage by rapidly killing trophonts before they caused their maximum damage.

We also observed stage-specific activity of HbβP-1 against *Amyloodinium ocellatum*. There was no apparent effect on either dinospores or tomonts at any HbβP-1 concentration. However, at high HbβP-1 concentrations, while infectivity of the dinospores and their subsequent differentiation into trophonts appeared normal at 48 hr, most trophonts did not differentiate into tomonts. This stage-specific activity would not only protect the infected individual from re-infection, but could also prevent spread of the infection, adding to herd immunity.

Stage-specific activity against *A. ocellatum* is also seen with the polypeptide antibiotics histone-like proteins (HLPs), isolated from the skin and gills of rainbow trout (*Oncorhynchus mykiss*), channel catfish and hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female)(Noga et al 2001, 2002). HLPs are closely related, if not identical to histones, with HLP-1 showing strong homology to histone H2B while HLP-2 is related to histone H1. Both HLP-1 and HLP-2 were cidal to *Amyloodinium* in the dinospore infectivity assay (Noga et al 2001). HLP-1 caused a significant reduction in the growth (size) of *Amyloodinium* trophonts. In addition, many parasites exhibited 'delayed mortality', where trophonts appeared to develop normally from dinospores but tomonts that differentiated from trophonts died before developing dinospores (Noga et al 2002). We observed a similar 'delayed mortality' in a few trophonts exposed to 50 and 100 μg/ml of HbβP-1 (i.e., formed tomonts but did not progress to dinospores), but not at 200 μg/ml.

Although *Amyloodinium* does not affect freshwater fish such as channel catfish, proteins homologous to HbβP-1 are present in microsomes of gill cells from a number of

teleosts including Mozambique tilapia (*Oreochromis mossambicus*), rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*), European eel (*Anguilla anguilla*) and elephant fish (*Gnathonemus petersii*) (Schuurmans Stekhoven et al 2004). The presence of Hb-β in microsomes from gill epithelium of primitive to advanced teleosts, some of which (trout and eel) spend part of their lives in sea water, suggests that Hb-β-derived AMPs like those isolated from the channel catfish may be expected even in marine fish. We have previously shown that HbβP-1 is highly active in physiological salt concentration and is cidal to ich under those conditions (Ullal et al, In preparation). Our present study further suggests that HbβP-1 may protect against marine ectoparasites. These results, combined with our findings of parasite-induced upregulation of HbβP-1 expression in skin and gill epithelium (Ullal et al, In preparation), suggest a critical role for these polypeptides in innate immunity to ectoparasites.

Piscidin 1 was lethal to ich trophonts at the same concentrations as HbβP-1, but it required a significantly longer time to kill trophonts (Table 3.2). Piscidin 1, isolated from hybrid striped bass mast cells (Silphaduang and Noga 2001), is a classical linear, highly cationic (pI 12.01; Compute pI/Mw tool at www.expasy.org), amphipathic, α-helical AMP and shows strong antibacterial as well as antiparasitic activity. In contrast, HbβP-1 is only slightly cationic (pI 8.49) but appears to have some amphipathic structure with flanking random coils at either terminal; it has weak antibacterial activity compared to its activity against protozoan ectoparasites (Ullal et al, In preparation). Parish et al (2001) also showed that an AMP derived from Human Hb-β was more lethal to certain fungi (*Candida albicans*) than most bacteria.

The main site of action of AMPs appears to be the cytoplasmic membrane, where they might assemble to form pores or channels, compromising the integrity of the phospholipid membrane. This is believed to occur either by AMPs inserting themselves through the membrane much like the staves of a barrel ("barrel-stave" model), oriented such that the hydrophilic region lines the central lumen and the hydrophobic side interacts with the lipid core of the bilayer or by forming a "toroidol-pore" by inducing the membrane phospholipids to bend in on themselves so that the water core is lined by both the peptide and the lipid head groups. An alternative hypothesis suggests that AMPs may cluster at the membrane surface, causing cooperative permeabilization of the cytoplasmic membrane via a "carpet effect" (Hancock and Lehrer 1998, Brogden 2005). Other mechanisms, exclusively based on studies with bacteria, have suggested that AMPs might traverse the cell membrane and influence intracellular targets by interfering with bacterial DNA and/or protein synthesis, or by stimulating autolytic enzymes (Boman et al 1993, Epand and Vogel 1999, Wu et al 1999). Amphipathic α-helical AMPs like piscidin 1 are believed to function according to the "barrel-stave" model. But the "carpet effect" may be likely for HbβP-1's action, at least against ich, since it's weak overall charge and random-coiled termini may not be conducive to transmembrane orientation as observed in more basic, amphipathic helices. Also, the discontiguous disintegration of the trophont's plasma membrane may be the result of HbβP-1 molecules aligning parallel to the bilayer, as compared to the relatively homogenous failure of membrane integrity that may be expected with the "barrel-stave" mechanism of AMPs like piscidin 1 (Brogden 2005).

There was a significant difference in the speed with which the two AMPs killed ich trophonts, with HbβP-1 acting almost twice as fast as piscidin 1 of comparable

concentrations (Table 3.2). The qualitative reaction of the parasites was also different: hyperactivity, encystment, excystment, and discontinuous cessation of ciliary motion were only seen with HbβP-1 (Fig. 3.1). Clark et al (1996) also observed hyperactivity followed by secretion of a mucoid cyst when trophonts were treated with a murine monoclonal antibody (mAb) recognizing the immobilization-antigen (I-Ag) on the trophont's surface. This mAb also induced premature exit of trophonts from the host. While further studies are needed, this suggests that in addition to a directly lethal effect, HbβP-1 may also induce the premature exit of ich. Based on the similar trophont responses to the I-Ag mAb and to HbβP-1, HbβP-1 may be interacting specifically with the I-Ag, although it is possible that hyperactivity and encystment are a non-specific avoidance response of the trophont and the modes of action of the two are unrelated. Also, catfish antisera against ich I-Ag induced agglutination of theronts (Clark et al 1988), which was not observed with HbβP-1 treatment. If HbβP-1 is interacting with I-Ag, it is likely not to be a "specific" interaction, but rather recognition of some general chemical structure. This might be analogous to recognition of LPS by certain AMPs as a pathogen-associated molecular pattern (PAMPs) (Medzhitov and Janeway 1997, Epand and Vogel 1999). Interestingly, the I-Ag has been identified in a number of ciliates (Clark et al 1995) and may be among the PAMPs that are believed to be recognized by the innate immune system and its AMP component.

HbβP-1 is not hemolytic to channel catfish erythrocytes (Table 3.2), nor is it cytotoxic to walking catfish gill (G1B) cells, indicating a very selective preference for the parasite's plasma membrane. It affects very different phyla (*Dinoflagellata* [*Amyloodinium*] and *Ciliophora* [*Ichthyophthirius*]), yet selectively targets the trophont of each. These findings support a role for HbβP-1 as a rapid and broad-spectrum yet still highly targeted

antiparasitic defense. HbβP-1 and related peptides in this family could also be expected to function in conjunction with the histone-like proteins that were isolated from the same epithelial tissues in fish, providing a strong line of innate host defense against eukaryotic, as well as prokaryotic pathogens.

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Table 3.1. Effect of trophont size on activity of HbβP-1 against *Ichtyophthirius multifiliis*. Nine trophonts (large or small) were tested at each HbβP-1 concentration. Pairwise comparisons were made of the time required to observe each change in small versus large trophonts. Values with different letter superscripts for an observed response (within a column) at the same HbβP-1 concentration are significantly different ($p < 0.0001$).

Legend

Encystment – Time when secretion of a gelatinous cyst wall began.

Excystment – Time when the parasite began to exit the cyst.

Cessation of ciliary motion begins – Time when ciliary motion began to stop. This typically began at focal areas on the cell, initially not affecting the entire cell.

Death – Time when all motility had ceased including all ciliary movement.

[+]One parasite (of 9 observed) encysted, then excysted

Table 3.1.

HbβP-1 Concentration in μg/ml (μM)	Trophont size in μm (mean ± SD)	Parasite response time (sec) at various stages of exposure (mean ± SD)			
		Encystment	Excystment	Cessation of ciliary motion begins	Death
25 (6.8)	Small (231 ± 23)	(25)[a+]	(40)[a+]	63 ± 13[a]	114 ± 15[a]
	Large (519 ± 58)	51 ± 9[b]	76 ± 11[b]	121 ± 33[b]	254 ± 48[b]
12.5 (3.4)	Small (222 ± 29)	-[a]	-[a]	61 ± 11[a]	95 ± 20[a]
	Large (500 ± 61)	49 ± 10[b]	81 ± 16[b]	99 ± 18[b]	171 ± 46[b]

Table 3.2. Killing rates of various HbβP-1 and piscidin 1 concentrations against *I. multifiliis* trophonts. Death was defined as complete loss of ciliary motion. Each value represents duplicate wells with three trophonts per well. Values at the same concentration with different letter superscripts are significantly different ($p < 0.0001$). Replication of this experiment yielded similar results.

Peptide Concentration	Time to death (mean ± SD)		Hemolytic activity*	
	HbβP-1	Piscidin 1	HbβP-1	Piscidin 1
12.5 μg/ml	313 ± 29^a sec	568 ± 23^b sec	0%	1.4%
25 μg/ml	238 ± 10^a sec	440 ± 14^b sec	0%	25.9%
50 μg/ml	218 ± 27^a sec	365 ± 37^b sec	0%	79.8%
100 μg/ml	167 ± 26^a sec	351 ± 35^b sec	0%	100%
200 μg/ml	165 ± 14^a sec	375 ± 29^b sec	0%	100%

Average size of trophont treated with HbβP-1 (323 μm) was not significantly different than that treated with piscidin 1 (318 μm) ($p = 0.0416$) as per Student's t-test at $\alpha = 0.01$

*Data from Ullal et al (In preparation).

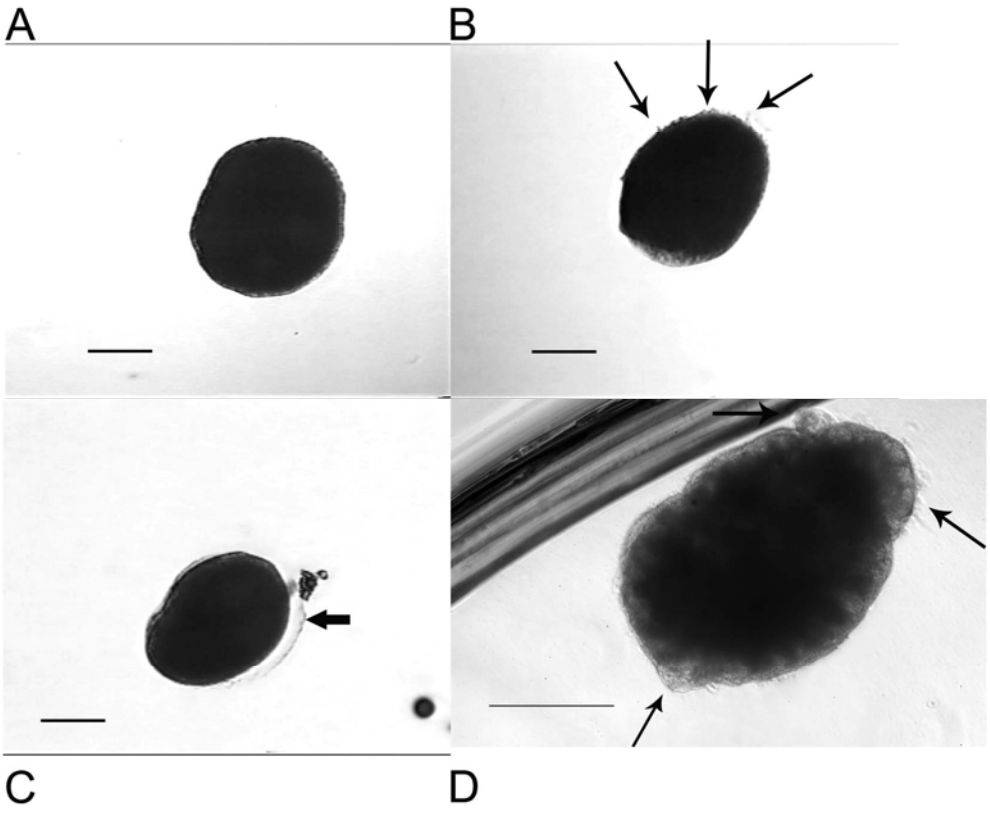
Figure 3.1. Cytological effects of of HbβP-1 and piscidin 1 on ich trophonts.

A. Intact untreated *I. multifiliis* trophont. Bar = 150 μm.

B. Ich trophont treated with 10 μM piscidin 1 for 400 sec. Note the reduced extent of cytoplasm leakage (arrows) as compared to HbβP-1 (D). Bar = 150 μm.

C. Ich trophont treated with 6.8 μM HbβP-1for 60 sec. Note the gelatinous cyst (arrow) secreted around the parasite. Secretion of a cyst was never observed in response to piscidin 1. Bar = 150 μm.

D. Ich trophont lysed following treatment with 6.8 μM HbβP-1 and excystment. Note the cytoplasm leaking out of the damaged cell membrane (arrows) indicating severe membrane damage. Bar = 150 μm.


A
B
C
D

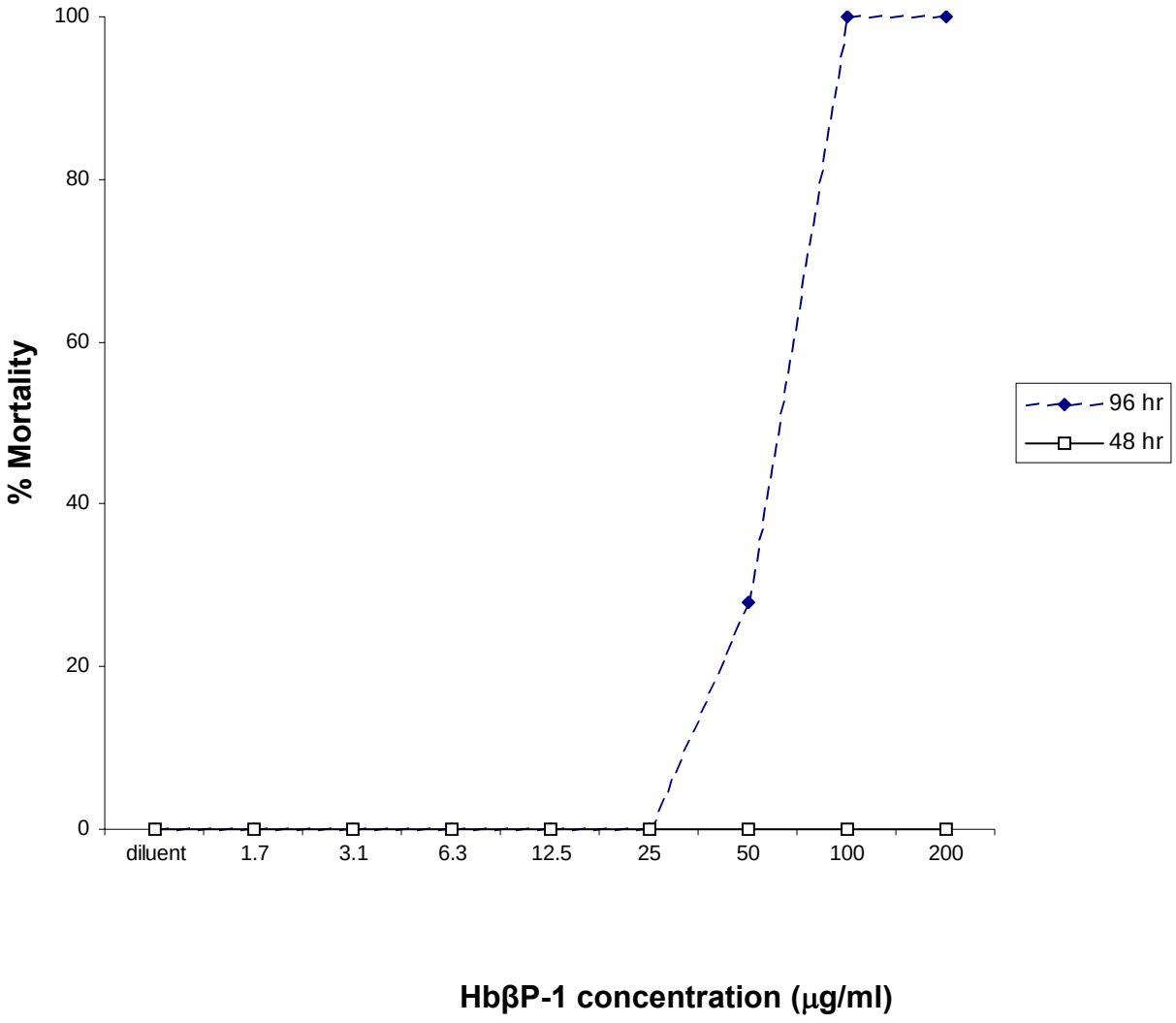


Fig 3.2. Mortality of *Amyloodinium ocellatum* after treatment for 96 hr with a range of HbβP-1 concentrations. Mortality is expressed as a percentage of sham-treated control cultures.