

Histone-like proteins from fish are lethal to the parasitic dinoflagellate *Amyloodinium ocellatum*

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(Received 1 September 2000; revised 25 January 2001; accepted 26 January 2001)

SUMMARY

Antimicrobial proteins were purified from acid extracts of rainbow trout (*Oncorhynchus mykiss*) and sunshine bass (*Morone saxatilis* male × *M. chrysops* female) skin, gill and spleen by reverse-phase HPLC. Mass spectrometry and amino acid sequence data suggest that these proteins are closely related to histone H2B and histone H1 and thus they were designated histone-like proteins (HLPs). These proteins were lethal to *Amyloodinium ocellatum*, which is one of the most important parasitic agents affecting fish. Antibiotic concentrations as low as 12.5 µg/ml were inhibitory. Activity was directed against the trophont (feeding) stage of the parasite, while the disseminative (dinospore) stage was unaffected. Thus, HLPs act unlike typical drugs used to treat amyloodiniosis, which usually target the dinospore. Both the ability of the parasite to infect host cells, as well as the ability to grow and differentiate after infection were severely inhibited. This is in contrast to magainin 2, which was similarly toxic to both the dinospore and trophont stages. These findings provide further evidence that histone-like proteins may be important defensive molecules in fish.

Key words: polypeptide antibiotics, fish, non-specific immunity, histones.

INTRODUCTION

As in other animals, parasites are a major cause of morbidity and mortality in fish (Schnick *et al.* 1997). Among the most important pathogens are the protozoan ectoparasites (gill and skin parasites), which are extremely common and cause serious losses in virtually all cultured marine and freshwater fish (Woo, 1995). Among the most important of the ectoparasitic protozoa is *Amyloodinium ocellatum*, a dinoflagellate which causes one of the most serious diseases of warmwater marine aquaculture. As aquaculture has developed worldwide, *Amyloodinium* has caused increasing losses in many species and is often the most important disease limiting warmwater marine aquaculture (Noga & Levy, 1995). *Amyloodinium* is an obligate parasite having a life-cycle in which the infective stage (dinospore) attaches to the host cell, forming the feeding stage (trophont), which damages the host. After feeding, the trophont detaches from the host cell, falls off the host, and forms the reproductive stage (tomont), which produces dinospores. *Amyloodinium ocellatum* is the only protozoan ectoparasite which can be propagated *in vitro*, and is a useful model for closely examining the effects of immune components on ectoparasite survival in a system which is very similar to that of the *in vivo* host (Noga, 1987; Smith *et al.* 1993; Oestmann & Lewis, 1996; Cobb, Levy & Noga, 1998).

Epithelial surfaces of fish, such as the skin and

gills, are composed of living, non-keratinized tissue covered by a layer of mucus. This living tissue is highly susceptible to colonization by pathogens. Fish probably rely heavily upon innate immunity for initial protection against colonization by infectious agents (Yano, 1996). Antibody and specific cell-mediated responses in fish are less diverse than those of mammals, and are also limited in response by temperature constraints on fish metabolism (Bly & Clem, 1992). There is an increasingly large body of evidence which suggests that endogenous, host-produced, polypeptide antibiotics ('endobiotics' as defined by Lehrer, Harwig & Ganz, 1994) are important components of innate immunity in many organisms. We have recently isolated histone-like proteins (HLPs) from channel catfish (*Ictalurus punctatus*) which exhibit broad-spectrum activity against pathogenic bacteria and water moulds (Robinette *et al.* 1998). We wished to determine if similar defences occurred in unrelated fish species and whether such antibiotics had activity against protozoan ectoparasites. As such, we looked for these antibiotics in 2 economically important fish, sunshine bass (*Morone saxatilis* male × *M. chrysops* female) and rainbow trout (*Oncorhynchus mykiss*) and determined if these polypeptides had activity against *A. ocellatum*.

MATERIALS AND METHODS

Purification of antibiotics

Clinically normal rainbow trout and sunshine bass ranging from 30 to 45 cm in length and 2 to 4 kg in

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weight were obtained from local farms and processed on site. Fish were euthanized by a blow to the head and decapitation and then maintained on ice throughout processing. The epidermis was scraped from the dermis with a scalpel, and the scrapings were placed into ice-cold 1% acetic acid. Gill and spleen were dissected from each fish and processed similarly. Following processing of 4–6 fish, the cellular material was adjusted to 2.5–3 times the volume of tissue by adding ice-cold 1% acetic acid. The suspension was then heated until boiling began, and then allowed to cool to room temperature. The material was then homogenized (Tissue Tearor, Biospec Products; Bartlesville, OK) at high speed for 2–3 min. This acid extract was centrifuged at 15000 g for 45 min at 4 °C, and the supernatant fraction containing antimicrobial activity was stored at –70 °C. This extract usually remained stable (retained strong activity indefinitely) at –70 °C.

Four volumes of tissue extract (generally 1–2 mg total protein/ml) were mixed with 1 vol. of 2 M sulfuric acid and stirred at 4 °C for 1 h. The supernatant was clarified via centrifugation at 12000 g for 20 min and then the supernatant was mixed with 4 vols of 95% ethanol and incubated at –20 °C for 24 h. The material was then centrifuged at 12000 g for 15 min and the precipitated protein was washed twice with 95% ethanol. The sample was then lyophilized to dryness and stored at –70 °C.

After reconstituting in 0.1% trifluoroacetic acid (TFA) in water, the sample was subjected to reverse-phase high performance liquid chromatography (RP-HPLC) using a 3.2 × 150 mm, 5 μm, 300 angstrom, butyl C₄ column (PolyLC, Columbia, MD) equilibrated with 7% acetonitrile/0.1% TFA. Activity was eluted with a linear gradient from 7% acetonitrile/0.1% TFA to 70% acetonitrile/0.1% TFA over 55 min at a flow rate of 1.0 ml/min on a Waters 600 HPLC System (Waters Inc., Milford, MA). The elution profile was monitored at 214 nm. Fractions of 1 ml were collected across the entire gradient, lyophilized, and reconstituted in 100 μl of 0.1% TFA or 0.01% acetic acid. Antibacterial activity was assessed using the *Escherichia coli* D31 spot assay (Zasloff, 1987).

Analysis of purity and protein identification

The purity of antimicrobial polypeptides isolated via RP-HPLC was assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970) using either the Mini-Protean II (BioRad; Richmond, CA) or PhastSystem (Pharmacia Biotech; Uppsala, Switzerland) electrophoresis systems according to manufacturer's instructions.

Confirmation that antimicrobial activity was associated with particular proteins was accomplished using a modification of a gel overlay assay (Blank

et al. 1983). Briefly, the sample was first resolved on a 15% Mini-Protean II Ready Gel (BioRad; Richmond, CA) using SDS–PAGE, with each sample being run in duplicate on each half of the gel. Cecropin A (isolated from the moth *Hyalophora cecropia*) (Sigma Chemical Co., St Louis, MO) was used as a positive antibacterial peptide control. After electrophoresis, the gel was split in half. One half was stained with Coomassie Blue and the other half was washed in 125 ml of 0.01 M Tris–HCl, pH 7.5/0.005 M 2-mercaptoethanol/25% isopropanol at 37 °C for 60 min with buffer changes every 20 min, followed by 2 × 15 min washings in 125 ml of 0.01 M Tris–HCl, pH 7.5 at 37 °C. The gel was then washed in 500 ml of 0.05 M Tris–HCl, pH 7.5/0.005 M 2-mercaptoethanol/0.001 M ethylenediaminetetraacetic acid (EDTA) for 18–24 h at 4 °C. Finally, the gel was washed in 125 ml of sterile 1 × Luria-Bertani broth/0.2 M phosphate buffer, pH 6.7/0.20% glucose/0.1 mg/ml streptomycin sulfate for 30 min at room temperature. The gel was 'blotted' by gently overlaying it onto an agarose plate inoculated with *E. coli* D31 (Zasloff, 1987), and the plate was incubated at 37 °C for 18–24 h. After incubation, zones of clearing beneath the blotted gel were compared with the stained section of gel to identify proteins demonstrating antibacterial activity.

The N-terminal amino acid sequences of purified polypeptides were determined via automated Edman degradation on an Applied Biosystems 477A pulsed-liquid protein sequencer, with the derivatized PTH (phenylthiohydantoin) amino acid analysis carried out with an on-line microbore Applied Biosystems 120A HPLC. Sequence data were analysed for homology to other known sequences using the Basic Local Alignment Search Tool (BLAST)(www.ncbi.nlm.nih.gov). The molecular masses of the purified antibacterial proteins were determined by matrix-assisted laser desorption mass spectrometry on a PerSeptive Biosystems Voyager® workstation (PerSeptive Biosystems, Framingham, MA).

Antiparasitic assays

Antiparasitic activity was first examined using our standard *in vitro* assay of *A. ocellatum* dinospore infectivity (Noga, 1989). Confluent monolayers of a fish gill cell line (GIB cells) were prepared in 96-well tissue culture plates; GIB monolayers were adapted to an artificial seawater solution (IO2/HBSS) before use in experiments. Infective dinospores were produced using standard procedures and 35 dinospores were inoculated into replicate wells having various antibiotic dilutions prepared in 0.01% acetic acid. The numbers of differentiated dinospores (i.e. trophonts and tomites) present in each well were counted under phase-contrast microscopy after incubation for 48 h at 25 °C. Values were expressed as a percentage of the sham-treated control (diluent

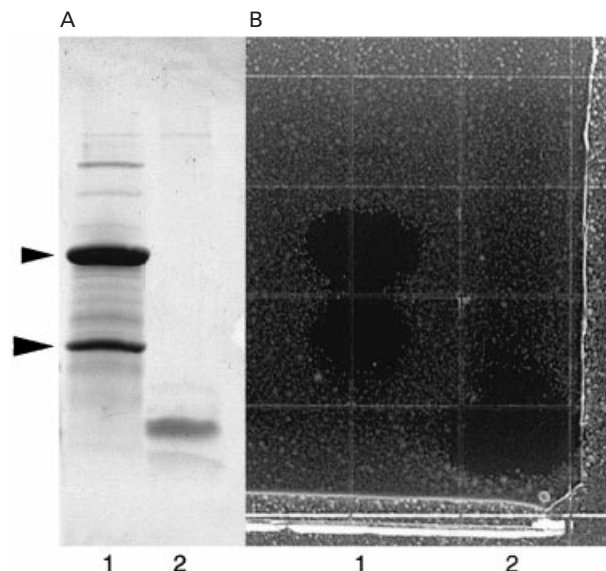


Fig. 1. SDS-PAGE of rainbow trout sulfuric acid/ethanol extract. (A) Gel section stained with Coomassie Blue R-250. Lane 1, major bands corresponding to HLP-1 (large arrow) and HLP-2 (small arrow); Lane 2, cecropin A. (B) Identical gel section overlaid onto an *E. coli* D31 plate. Note that an empty lane separates Lanes 1 and 2 on this gel to prevent cross-over of clearing zones into adjacent lanes.

only added). As a putative positive control, we used magainin 2 (Sigma Chemical Co., St Louis, MO), a peptide antibiotic isolated from aquatic frogs (*Xenopus laevis*), which has antiprotozoal activity (Zasloff, 1987). We also examined the activity of purified calf histone H2B (Boehringer-Mannheim, Raleigh, NC).

To determine if antibiotics were specifically toxic to trophonts, anti-trophont activity was assessed by inoculating replicate wells with 35 dinospores. After allowing the dinospores to infect the monolayer for 30 min and subsequently differentiate into trophonts, the cultures were washed 5 times to remove any free-swimming dinospores, leaving only attached trophonts. Various antibiotic treatments (all 100 $\mu\text{g/ml}$) were then added. The numbers of visible trophonts (i.e. viable parasites which infected the cell culture and grew) were counted after 48, 72, and 96 h. Values were expressed as a percentage of the sham-treated control (diluent only).

The effect of various antibiotics on dinospores was assessed by mixing 35 dinospores with an equal volume of antibiotic solution and then incubating the suspension at 25 °C for 60 min. The suspension was then added to replicate wells having G1B cells in IO2/HBSS. In this manner, the initial antibiotic concentration to which the dinospores were exposed for 60 min was diluted 10-fold from 100 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$ in the wells (centrifuging dinospores to concentrate them causes a large loss of infectivity, possibly due to loss of flagella). As a control, dinospores were treated similarly except that they

were only exposed to the antibiotic diluent (0.01 % acetic acid). To account for any possible residual effect of the diluted antibiotic which was present in the culture wells, a separate group of dinospores was exposed only to the diluted antibiotic treatments after being added to the wells with G1B cells (i.e. no exposure while being incubated for 60 min prior to adding to the wells). This was the sham-treated dinospore control.

Data were analysed as a 2 factorial design with repeated measurements in time (Neter, Wasserman & Kutner, 1990). One factor was type of antibiotic; the other factor was life-stage. These two factors were compared at all 3 time-intervals for which data were collected (48, 72 and 96 h after adding parasites to the G1B cultures). For each possible combination of these 2 factors, the measurement was replicated 3 times (i.e. 3 wells). Since all data were expressed as a percentage of the control (diluent only), arcsine transformation was done to normalize the data (Zar, 1996). All analyses were done using PROC GLM in SAS version 7 (SAS Institute, Cary, NC).

RESULTS

Purification of HLPs

Since our previous data indicated that HLPs were highly basic molecules (Robinette *et al.* 1998), we produced a large amount of tissue extract in 1 % acetic acid for further purification and we exploited a method that was similar to that used in purifying histones (Moehs *et al.* 1992; Johns, 1977). Following sulfuric acid extraction and ethanol precipitation, part of the precipitate was dissolved in 0.01 % acetic acid and run on SDS-PAGE. There were 2 major bands in this material (Fig. 1). The SDS-PAGE overlay assay revealed discrete clearing zones corresponding to the position of the predominant polypeptides (Fig. 1).

The rainbow trout and sunshine bass sulfuric acid/ethanol extracts were fractionated using RP-HPLC, resulting in peaks having antibacterial activity. The major antibacterial activity in the trout extract (Peak I), eluting at approximately 55 % acetonitrile, was purified to homogeneity as was the other major polypeptide eluting at about 45–47 % acetonitrile (Fig. 2). Similar results were obtained with sulfuric acid/ethanol extracts from sunshine bass (Fig. 2). The major antibacterial peak in the bass extract (Peak I) eluted at approximately 50 % acetonitrile, while Peak II eluted at about 35–40 % acetonitrile. In bass, Peak II was much less abundant than Peak I and was not further characterized.

Mass spectrometry indicated that the molecular mass of the major rainbow trout antibacterial protein was 13489.2 Da. A partial N-terminal amino acid sequence yielded PEPAKSAPKKGSKKAVT, which had 100 % homology with rainbow trout histone H2B (Kootstra & Bailey, 1978). The mol-

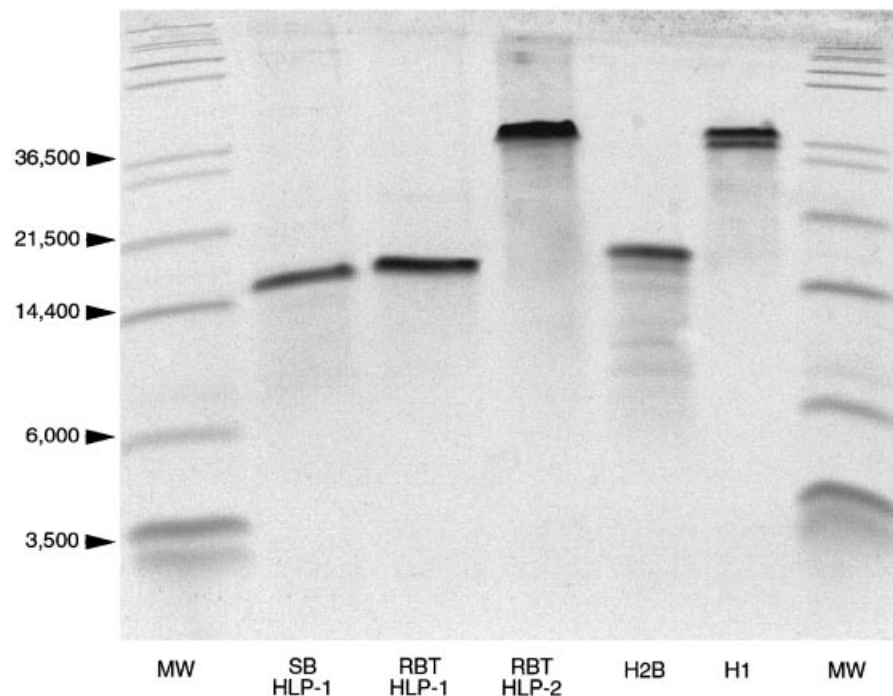


Fig. 2. Tris-tricine SDS-PAGE of the antibacterial proteins isolated from rainbow trout and sunshine bass purified by reverse-phase HPLC. MW molecular weight markers; SB HLP-1, sunshine bass HLP-1; RBT HLP-1, rainbow trout HLP-1; RBT HLP-2, rainbow trout HLP-2; H2B, calf histone H2B; H1, calf histone H1. (1 μ g protein loaded in each lane.)

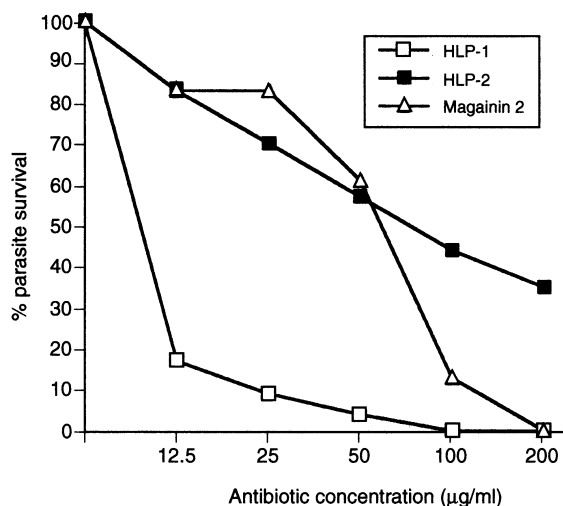


Fig. 3. Antiparasitic activity of HLP-1, HLP-2 and magainin 2 against *Amyloodinium ocellatum* using the dinospore infectivity assay. Replication of this experiment yielded similar results.

ecular mass of the next most abundant antibacterial protein isolated from RP-HPLC (peak II) was 20714.9 Da as determined by mass spectrometry (data not shown); a partial N-terminal sequence yielded AEVAPAPAAAAPAKAPKKKA. This sequence had 100% homology with rainbow trout histone H1 (Macleod, Wong & Dixon, 1977). Due to their similarity to histones and to HLPs isolated from channel catfish (Robinette *et al.* 1998), the trout antibacterial proteins were designated rainbow trout

Histone-Like Proteins (HLP) 1 and 2, corresponding to similar polypeptides isolated previously from channel catfish (Robinette *et al.* 1998). Extracts from sunshine bass had antibacterial activity which was mainly due to a 13737.6 Da polypeptide, which after RP-HPLC purification, yielded a sequence having approximately 85% PDPAPKTAPKKGSKKAV-TKTAG; ~15% of the sequence was identical to this sequence except for the lack of 2 terminal residues. This polypeptide also had high sequence homology to histone H2B (Kootstra & Bailey, 1978) and was thus designated as sunshine bass HLP-1.

Anti-Amyloodinium activity

Both HLP-1 and HLP-2 were cidal to *A. ocellatum* in the dinospore infectivity assay (Fig. 3). Death was confirmed by the fact that in cultures with 0% infectivity, no viable parasites were observed for 1 month after beginning the experiment. Cultures treated with HLP-1 or calf histone H2B typically had much smaller trophonts and tomonts which often failed to reinfect the G1B monolayer, unlike control (diluent only) cultures in which most of the dinospores produced large trophonts and subsequently many dinospores. In cultures exposed to magainin 2, an especially large number of the tomonts died, as evidenced by a shrinkage of the cytoplasm away from the cyst wall. On a weight basis, HLP-1 was more potent than magainin 2, while HLP-2 was similar in potency to magainin 2. On a molar basis, both HLP-1 and HLP-2 were

Table 1. Toxicity of polypeptide antibiotics to dinospores and trophonts of *Amyloodinium ocellatum*

(Values are the number of trophonts and tomonts present expressed as a percentage of the control cultures (diluent only). Percentages are mean (\pm s.e.) values for triplicate wells counted at 48, 72 and 96 h after adding parasites to the cultures. All antibiotic treatments of all 3 life-stage treatments at all 3 time-periods were significantly different ($P < 0.01$) from control cultures of the same life-stage treatment at the same time period. Replication of this experiment yielded similar results.)

Treatment	Dinospores sham-treated			Dinospores treated			Trophonts treated		
	48 h	72 h	96 h	48 h	72 h	96 h	48 h	72 h	96 h
Rainbow trout HLP-1	14 \pm 4	46 \pm 5	50 \pm 3	12 \pm 5	29 \pm 13	38 \pm 11	6 \pm 1	3 \pm 2	0 \pm 0
Sunshine bass HLP-1	58 \pm 7	65 \pm 11	65 \pm 16	36 \pm 6	50 \pm 21	54 \pm 23	21 \pm 1	22 \pm 4	16 \pm 8
Calf histone H2B	60 \pm 13	67 \pm 16	72 \pm 14	53 \pm 12	59 \pm 12	56 \pm 11	13 \pm 3	11 \pm 4	4 \pm 2
Magainin 2	50 \pm 5	51 \pm 11	54 \pm 6	64 \pm 11	54 \pm 5	52 \pm 4	42 \pm 2	40 \pm 2	43 \pm 2

Table 2. Relative effect of various antibiotics on the 3 life-stage treatments

(For each antibiotic at every time-interval (48, 72, 96 h), each life-stage treatment was compared with the other 2 life-stage treatments. Underlined values are significant ($P < 0.01$). RBT HLP-1, rainbow trout HLP-1; SB HLP-1, sunshine bass HLP-1; H2B, calf histone H2B; MAG2, magainin 2; D, dinospore; DS, dinospore-sham; T, trophont.)

	RBT HLP-1			SB HLP-1			H2B			MAG2		
	48	72	96	48	72	96	48	72	96	48	72	96
D v. DS	0.7077	0.2226	0.4962	0.0295	0.4032	0.7681	0.4053	0.3035	0.1678	0.1348	0.8635	0.9121
D v. T	0.3135	0.02	<u>0.0011</u>	0.0799	0.0573	<u>0.0072</u>	<u>0.0001</u>	<u>0.0019</u>	<u>0.0007</u>	0.0245	0.4058	0.5984
DS v. T	0.1707	<u>0.0009</u>	<u>0.0002</u>	<u>0.0003</u>	<u>0.0083</u>	<u>0.0034</u>	<u>0.0001</u>	<u>0.0001</u>	<u>0.0001</u>	0.4125	0.5082	0.5274

Table 3. Statistical comparison of the activity of various antibiotics on the life-stage treatment

(For each life-stage treatment at every time-interval, each antibiotic was compared with the other 3 antibiotics. Underlined values are significant ($P < 0.01$). RBT, rainbow trout HLP-1; SB, sunshine bass HLP-1; G2B, calf histone H2B; Mag, Magainin 2; D, dinospore; DS, dinospore-sham; T, trophont.)

	48 h			72 h			96 h		
	SB-D	H2B-D	Mag-D	SB-D	H2B-D	Mag-D	SB-D	H2B-D	Mag-D
RBT-D	<u>0.0035</u>	<u>0.0001</u>	<u>0.0001</u>	0.1193	0.0459	0.0982	0.1851	0.3264	0.4378
SB-D		0.0868	<u>0.006</u>		0.6354	0.9184		0.7224	0.573
H2B-D			0.2443			0.7098			0.834
	SB-DS	H2B-DS	Mag-DS	SB-DS	H2B-DS	Mag-DS	SB-DS	H2B-DS	Mag-DS
RBT-DS	<u>0.0001</u>	<u>0.0001</u>	<u>0.0002</u>	0.2372	0.0693	0.7752	0.3423	0.0959	0.8361
SB-DS		0.7452	0.3936		0.503	0.3659		0.4566	0.4554
H2B-DS			0.242			0.121			0.1414
	SB-T	H2B-T	Mag-T	SB-T	H2B-T	Mag-T	SB-T	H2B-T	MAG-T
RBT-T	0.0238	0.231	<u>0.0001</u>	0.0458	0.2696	<u>0.0024</u>	0.0452	0.4165	<u>0.0005</u>
SB-T		0.2568	0.0227		0.3455	0.2255		0.2151	0.0848
H2B-T			<u>0.0013</u>			0.036			<u>0.0048</u>

much more potent than magainin 2 (MW = 2466.9). In addition, the activity displayed by magainin 2 was sometimes accompanied by non-specific cytotoxicity, since in some replications, G1B cells were observed to be detaching in cultures having magainin 2 concentrations of 50 μ g/ml or higher. No G1B cytotoxicity was observed in any cultures treated with HLP-1, HLP-2, or calf histone H2B.

Exposure of *A. ocellatum* to all 4 antibiotics (rainbow trout HLP-1, sunshine bass HLP-1, calf histone H2B, magainin 2) caused a significant ($P < 0.01$) decrease in the number of parasites in all 3 life-stage treatments (dinospore-sham, dinospore, trophont) at all 3 time-intervals (48, 72, 96 h) (Table 1). However, there was no significant ($P > 0.01$) difference between dinospore versus dinospore-sham

treated groups for any antibiotic at any time-interval (Table 2). In contrast, there was a significant difference between both dinospore versus trophont, as well as dinospore-sham versus trophont, for rainbow trout HLP-1, sunshine bass HLP-1 and calf histone H2B. There was no difference between dinospore-sham versus trophont, nor between dinospore versus trophont, in magainin 2 (Table 2). Rainbow trout HLP-1 was significantly ($P < 0.01$) more potent than all 3 other antibiotics in affecting the dinospore and dinospore-sham groups, but was only significantly different from magainin 2 against trophonts (Table 3). Calf H2B was also more potent than magainin 2 in inhibiting trophonts. No other treatment comparisons were significantly different ($P > 0.01$).

DISCUSSION

Rainbow trout and sunshine bass skin, gill and spleen have proteins with potent antimicrobial activity. These histone-like proteins (HLPs) are closely related, if not identical to histones. The reported molecular weight of trout histone H2B is 13270 Da (Kootstra & Bailey, 1978), which strongly suggests that HLP-1 is highly related if not identical to histone H2B. The N-terminal sequence and molecular weight of sunshine bass HLP-1 also suggests that it is highly similar to histone H2B. However, the reported molecular weight of trout histone H1 (determined from complete sequencing of the molecule) is 19314 Da (Macleod *et al.* 1977). Thus, HLP-2 is probably related to histone H1, but may not be the same molecule. Confirmation of the exact identity of all of these molecules must await determination of their entire sequences.

Only recently (Robinette *et al.* 1998) have histone-like polypeptides been reported to be defensive molecules in fish. Park *et al.* (1998b) recently found that experimental trauma to an Asian catfish (*Parasilurus asotus*) induced the release of a potent antimicrobial peptide, parasin I, which was highly homologous to the N-terminal portion of histone H2A. However, previous research suggests that histones or histone-like molecules may play an important role in the defence of other vertebrates. A potent, broad-spectrum, polypeptide from the stomach of the Asian toad (*Bufo bufo gargarizans*) is a proteolytic cleavage product of histone H2A (Kim *et al.* 1996). Kashima (1991) found that histone H1 isolated from human epidermis had potent antifungal (candidacidal) activity. Hiemstra *et al.* (1993) found that polypeptides related to both histone H2B and histone H1 were present in the lysosomes of mouse macrophages. These antibiotics had potent, broad-spectrum activity against bacteria and fungi. Histones have also been recently isolated from a number of host compartments in mammals, suggesting that they may have other important roles beyond their

classical role as a structural component of the nucleosome (see Robinette *et al.* 1998 for a review of the recent literature).

Further evidence that HLPs are important defensive molecules in fish is provided by the fact that the relatively mild extraction techniques which we use to obtain HLP-1 and HLP-2 from fish tissues are not strong enough to release histone bound to nucleic acid (Moehs *et al.* 1992). Also, the activity is just as strong when we extract tissues in water (Robinette *et al.* 1998; E Noga and Z Fan, unpublished data). Thus, the polypeptides which we are isolating are free within the cell and/or extracellular space, as has been demonstrated for histone HI in human epidermis (Kashima, 1991).

Most importantly, we have found that HLP-1 and HLP-2 possess potent activity against important fish pathogens including bacteria and water moulds (Robinette *et al.*, 1998). Both HLP-1 and HLP-2 are lethal to *A. ocellatum*, one of the most important parasites of fish (Noga & Levy, 1995). While not an important ectoparasite of salmonids, *Amyloodinium* is an important pathogen of many other marine/estuarine fish such as sunshine bass (Noga, Landsberg & Smith, 1991). Rainbow trout HLP-1 is more potent than magainin 2, which is known to have significant antiprotozoal activity (Zasloff, 1987). We have previously shown that fish skin mucus is inhibitory to *Amyloodinium* via the dinospore infectivity assay (Landsberg *et al.* 1992). The presence of HLPs may explain this activity.

Both rainbow trout and sunshine bass HLP-1 can kill trophonts. The trophont stage would seem to be the most energetically efficient target of attack, since this stage is in the most intimate contact with the host and thus would be exposed to the highest concentration of antibiotic for the longest period of time. Dinospores rapidly differentiate into trophonts when they contact the host, typically within 5–20 min (Noga, 1987) and thus there would be much less time available to affect the dinospore. Interestingly, virtually all drugs/chemicals used to treat amyloodiniosis (e.g. copper or formalin) are only effective against the free-swimming, infective (dinospore) stage (Paperna, 1984; Noga & Levy, 1995), while the trophont stage is highly resistant to drug therapy.

There was no difference between the dinospore versus the dinospore-sham groups in parasites treated with either rainbow trout HLP-1, sunshine bass HLP-1, or calf histone H2B. This suggests that all of the inhibition seen in these two dinospore treatment groups was due to the presence of residual antibiotic which was transferred into the cultures when the dinospores were added to the cultures after the 60 min antibiotic exposure. These data, combined with the significantly greater inhibition of trophonts compared to either dinospores or dinospore-sham groups suggests that the trophont is the

main stage targeted by HLP-1 and histone H2B. In contrast, the toxicity of magainin 2 is similar in trophont, dinospore-sham and dinospore groups, suggesting that its activity is less targeted at a specific life-stage but rather can be toxic to both trophonts and dinospores.

Recently, other types of novel polypeptide antibiotics have been isolated from other fish, including hagfish (*Myxine glutinosa*, *Epatretus burgeri*), carp (*Cyprinus carpio*), loach (*Misgurnus anguillicaudatus*), and winter flounder (*Pleuronectes americanus*) (Lemaitre *et al.* 1996; Shinnar *et al.* 1996; Cole, Weis & Diamond, 1997; Park *et al.* 1997; Seo *et al.* 2000). In addition, lysozyme, which has been recognized as a component of fish blood and body secretions for some time, is also antibacterial (Grinde, 1989). However, none of these polypeptide antibiotics from fish have been shown to possess the spectrum of activity (bacteria, water moulds, parasites) of HLPs (Robinette *et al.* 1998).

In recent years, a number of studies have shown that other polypeptide antibiotics can be toxic to various parasites, including trypanosomes (Durvasula *et al.* 1997), *Leishmania* (Diaz-Achirica *et al.* 1998), *Plasmodium* (Shahabuddin *et al.* 1998), *Giardia* (Aley *et al.* 1994) and amoebae (Huang, Chen & Zierdt, 1990). Parasites of aquatic animals, such as *Bonamia ostreae* (the cause of bonamiosis) and *Perkinsus marinus* (the cause of 'dermo'), are also susceptible to endobiotics (Morvan *et al.* 1994; Pierce *et al.* 1997).

The main site of action of the majority of endobiotics is believed to be the plasma membrane, where they cause pore formation or membrane lysis (Hancock & Lehrer, 1998). The selectivity for 'foreign' membranes such as those of bacteria, is believed to result from high content of anionic lipids on the surface of the bacterial membrane, high potential gradient across the membrane and lack of cholesterol (Hancock & Lehrer, 1998). Presumably, similar properties are responsible for parasite selectivity. Potency of most linear polypeptide endobiotics is related to their ability to form an amphipathic α -helix. For example, the most active antiparasitic magainin analogues have the greatest α -helical structure (Huang *et al.* 1990). The mechanism responsible for the toxicity of HLPs to *Amyloodinium* is unknown. However, buforin II, an antimicrobial polypeptide related to histone H2A of the toad *Bufo bufo gargarizans*, presumably kills bacteria by entering and accumulating in the bacterial host cell and binding to its nucleic acids (Park, Kim & Kim, 1998a). Despite the amphipathic α -helical structure of buforin II, it was not lytic to bacteria. The chemistry and structure of the dinoflagellate nucleus is quite different from that of other eukaryotes (Taylor, 1987), and thus may present a specific target for HLPs. Interestingly, dinoflagellates package the majority of their DNA with small basic proteins

totally unrelated to histones (Vernet *et al.* 1990). While the mechanism of action of HLPs remains to be elucidated, their potent activity against *A. ocellatum*, combined with their isolation from widely divergent fish species (rainbow trout, sunshine bass, channel catfish) suggests that they may be a very important defence against ectoparasite infections of fish. Thus, HLPs and other endobiotics may prove to be a very rich source of anti-parasitic strategies in the future.

We thank D. Robinette and M.-S. Yang for technical assistance, J. Carlton (Louisiana State University Core Laboratory) and M. Gawinowicz (Howard Hughes Medical Institute, Columbia University) for assistance with protein sequencing and mass spectrometry, D. Jennings (Jennings Trout Farm, Canton, NC) for supplying rainbow trout and L. Brothers (Carolina Fisheries, Aurora, NC) for supplying sunshine bass. This work was supported by Grant NA46RG0087 from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, to the North Carolina Sea Grant College Program, Saltonstall-Kennedy Project #NA67FD0131, and Research Grant No. US-3030-98 from BARD, the United States-Israel Binational Agricultural Research and Development Fund to E.J.N.

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