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Research Article

Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like antimicrobial proteins

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Abstract. Three antibacterial proteins were isolated from acid extracts of channel catfish (*Ictalurus punctatus*) skin by cation exchange chromatography and reverse-phase high-pressure liquid chromatography. The molecular masses of the proteins were 15.5, 15.5 and 30 kD as determined by SDS-polyacrylamide gel electrophoresis. Mass spectrometry, amino acid composition and amino acid sequence data suggest that the most abundant protein is closely related to histone H2B. The H2B-like protein was inhibitory to *Aeromonas hydrophila* and *Saprolegnia* spp., which are important bacterial and fungal pathogens of fish. These findings suggest that histones may be important defensive molecules in fish.

Key words. Antimicrobial proteins; catfish; non-specific immunity; histones.

The immune responses of lower vertebrates such as teleost fish are significantly less sophisticated than those of higher animals. Fish rely heavily upon innate or nonspecific immune mechanisms for initial protection against infectious agents. Antibody and specific cell-mediated responses in fish are less diverse than those of mammals [1] and are also limited in response time by temperature constraints on fish metabolism [2, 3]. The epithelial surfaces of fish, such as the skin, gills and alimentary tract, provide first contact with potential pathogens. These surfaces are composed of living, nonkeratinized tissue covered by a layer of mucus which contains a number of nonspecific antimicrobial factors, such as lysozyme, transferrin and C-reactive protein [4]. The fact that fish are normally capable of effectively controlling the resident flora and limiting infection, even though specific immune responses may be slow to respond, suggests that factors which can rapidly protect against a variety of infectious agents may be critically important in fish immunity.

There is an increasingly large body of evidence which suggests that endogenous antibiotics are important components of innate immunity in many organisms [5]. The vast majority of these factors are relatively low molecular weight peptides or proteins, such as the de-

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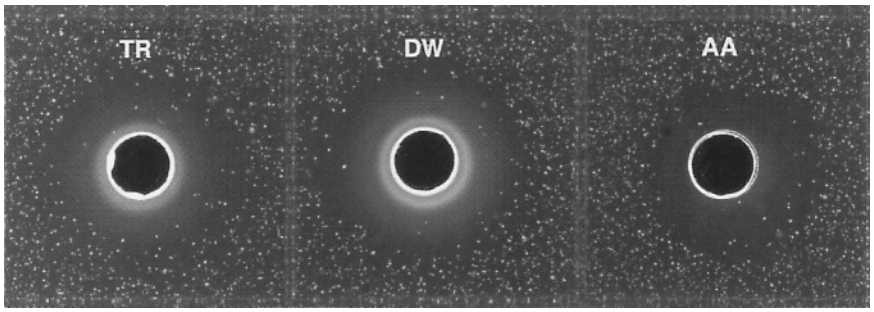

Figure 1. Comparison of the antibacterial activity of catfish skin extracts using the radial diffusion assay. Extracts were prepared in either 50 mM Tris buffer pH 7.2 (TR), distilled-deionized water (DW) or 1% acetic acid (AA).

fensins [6, 7], magainins (from *Xenopus* skin; [8]) and cecropins (from the hemolymph of the cecropia moth *Hyalophora cecropia*; [5, 9]). These peptides are often active against a wide range of microorganisms, including bacteria, viruses, fungi and protozoan parasites. Such broad-spectrum peptide and polypeptide antibiotics offer the distinct advantages of nonspecificity and rapid response, which allow the host to delay or avoid microbial colonization, factors which would be especially advantageous for fish. Lemaitre et al. [10] reported the isolation of two antibacterial proteins of 27 and 31 kD from the skin mucus of carp (*Cyprinus carpio*). In contrast to the majority of reported anti-microbial peptides and proteins, which are cationic, these factors were purified in part using anion-exchange chromatography, indicating an acidic nature. Lysozyme, and the aminosterol antibiotic squalamine, isolated from the dogfish shark (*Squalus acanthias*), represent the only other endogenous antibiotics thus far reported and characterized in fish [11]. Initial studies in our laboratory indicated the presence of significant antibacterial activity in the skin of channel catfish, *Ictalurus punctatus*, an economically important aquaculture species. The present study was undertaken to isolate and characterize the factor(s) in channel catfish skin responsible for this activity. The characterization of these factors will enhance our understanding of the mechanisms whereby fish resist infection. A more complete understanding of nonspecific immunity in fish may in turn enable the development of methods to enhance immune processes and protect valuable aquaculture species from infectious disease.

Materials and methods

Extraction and purification procedures. Initial studies in our laboratory indicated that skin extracts from channel catfish had significant antibacterial activity. The antibacterial activity of extracts prepared under different conditions was assessed in a radial diffusion assay [12] in which 2 µl of extract was pipetted into a well of an agarose plate having a suspension of *Escherichia coli* D31. After overnight incubation at 37 °C, the clearing zone diameter for each of the extracts was compared to determine if extraction in weak acid liberated more antibacterial activity than extraction under the more physiological conditions of either deionized water (ddH_2O) or 50 mM Tris buffer (pH 7.2). The potency of the activity, as judged by clearing zone diameters, was similar after equal amounts of skin were extracted with either ddH_2O, Tris buffer, or after extracting in dilute (1%) acetic acid and boiling for 15 s (fig. 1). Since our initial studies had indicated that the activity was cationic, we used the latter treatment to prepare extracts for purification.

Clinically normal fish measuring 30–45 cm in length and 2–4 kg in weight were obtained from a local farm and transported to the laboratory. Fish were maintained in a 1000-l aquarium. Fish were anaesthetized with tricaine 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% glacial acetic acid. Following processing and euthanasia of 4 to 6 fish, the cellular material was adjusted to 2.5 to 3 times the volume of tissue by adding ice-cold 1% glacial 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, USA) at high speed for 2 to 3 min. This acid extract was centrifuged at 15,000*g* for 45 min at 10 °C, and the supernatant containing antimicrobial activity was stored at −70 °C. This extract usually remained stable (retained strong activity) for at least 1 year when stored at −70 °C. The pellet was reextracted in 10 ml of ice-cold 1% glacial acetic acid by vortexing until the pellet was resuspended. This material was allowed to stand on ice for 30 min, and then centrifuged at 15,000*g* for 45 min at 10 °C. The supernatant was removed and stored at −70 °C. Reextraction of the

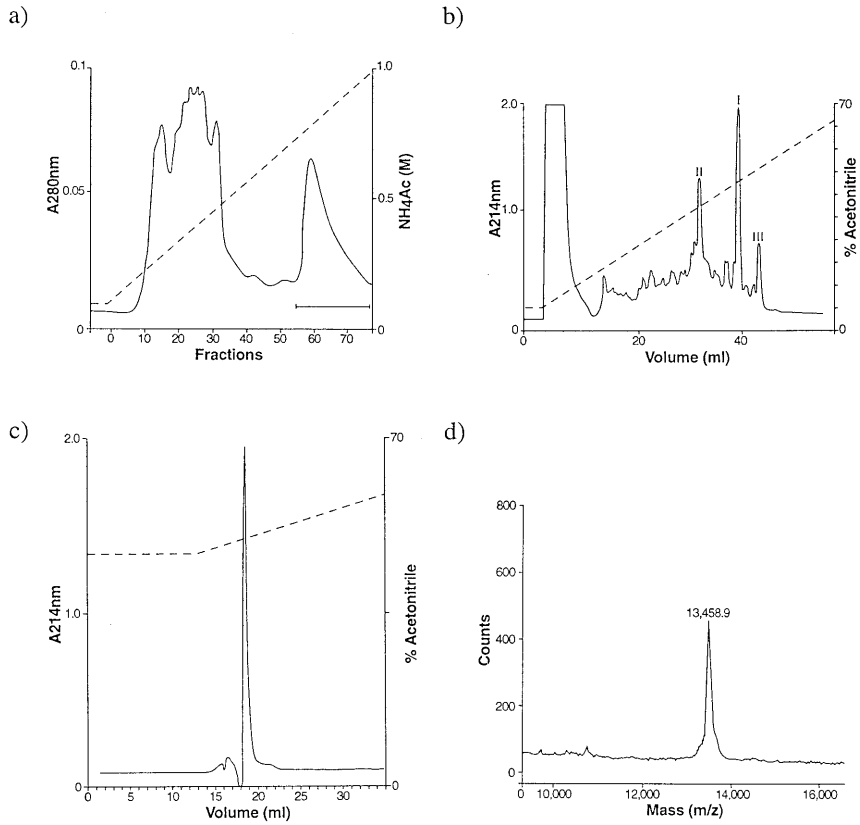

Figure 2. (*a*) CM52 cation-exchange chromatograph of crude catfish skin extract. Dashed line represents gradient of ammonium acetate buffer. Fractions containing antibacterial activity are denoted by the solid line. (*b*) C_4 reverse-phase HPLC chromatograph of antibacterial activity isolated from CM52 chromatography. Dashed line represents acetonitrile/0.1% TFA gradient. I, II and III denote antibacterial proteins in order of predominance. (*c*) Recycling of protein from peak 1 on C4 reverse-phase HPLC. (*d*) Mass spectroscopic analysis of RP-HPLC peak 1 purified protein.

resulting pellet was repeated in this manner until antimicrobial activity could no longer be recovered (generally 3–4 reextractions).

Skin extract (generally 100–200 mg total protein) was equilibrated with an equal volume of 0.1 M ammonium acetate/acetic acid buffer, pH 5.15 (AA buffer), and applied at a flow rate of 1 ml/min to a 16 × 200 mm column containing carboxymethylcellulose CM52 (Whatman Labsales, Hillsboro, OR, USA) equilibrated with AA buffer. Column eluate was monitored at 280 nm. After application of sample, the column was washed extensively with AA buffer until the absorbance returned to baseline, and then activity was eluted with a 300-ml gradient from 0.1 to 1.0 M AA buffer, pH 5.15. Fractions of 4 ml were collected across the entire gradient, lyophilized, reconstituted in 0.1 ml of 0.1 M AA buffer and assayed for activity using the *E. coli* D31 spot assay [8]. Fractions with antibacterial activity were pooled and lyophilized to concentrate the activity for further purification.

Cationic antibacterial activity was subjected to reverse-phase high-pressure liquid chromatography (RP-HPLC). The lyophilized activity recovered from one CM52 column run was reconstituted in 2 to 4 ml of 0.1% trifluoroacetic acid (TFA) in water, and injected onto a 3.2 × 150 mm, 5 μm, 300 Å, butyl C_4 column (PolyLC, Columbia, MD, USA) 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, Milford, MA, USA). The elution profile was monitored at 214 nm. Fractions of 1 ml were collected across the entire gradient, lyophilized and reconstituted in 0.1 ml of 0.1% TFA. Antibacterial activity was assessed using the *E. coli* D31 spot assay. Fractions representing discrete zones of activity corresponding to major chromatogram peaks were pooled and cycled once again through the C_4 column using a shallower gradient to obtain single peaks associated with antimicrobial activity.

Analysis of purity and protein identification. The purity and apparent molecular mass of antibacterial proteins isolated from RP-HPLC were assessed by SDS-polyacrylamide gel electrophoresis (PAGE) [13] using either the Mini-Protean II (BioRad, Richmond, CA, USA) or PhastSystem (Pharmacia Biotech, Uppsala, Sweden) electrophoresis systems according to manufacturer's instructions.

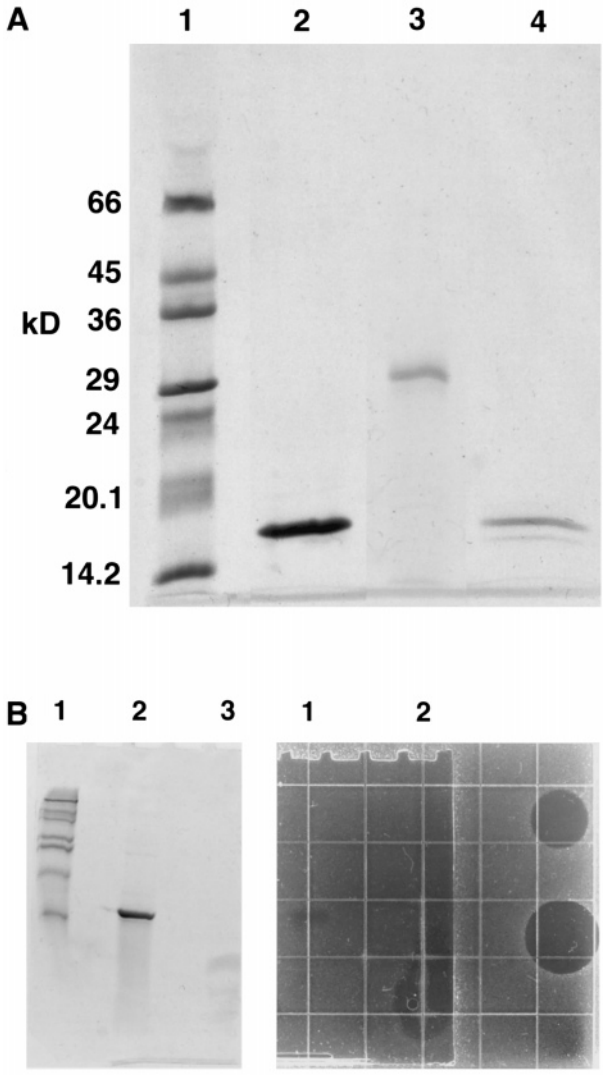

Figure 3. (*A*) SDS-PAGE analysis of the three antibacterial proteins isolated from reverse-phase HPLC. Lane 1, molecular weight markers; lane 2, RP-HPLC peak 1; lane 3, RP-HPLC peak 2; lane 4, peak 3. (*B*) SDS-PAGE gel overlay assay (bug blot) analysis of the major catfish antibacterial protein. (Left) Gel section stained with Coomassie Blue R-250. Lane 1, molecular weight markers; lane 2, 5 μg of HPLC-purified material; lane 3, 1.5 μg of cecropin A. (Right) Identical gel section overlayed onto an *E. coli* D31 spot assay plate. Lane 1, 5 μg of HPLC-purified material; lane 2, 1.5 μg of cecropin A. (Far right) Clearing zones associated with surface application of 5 μg of HPLC purified material (top) or 1.5 μg of cecropin A (bottom).

Confirmation that antibacterial activity was associated with particular proteins was accomplished using a modification of a gel overlay assay [14]. Briefly, the sample was first resolved on a 15% Mini-Protean II Ready Gel (BioRad, Richmond, CA, USA) using SDS-PAGE, with each sample being run in duplicate on each half of the gel. 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 two 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 to 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 6.7/0.2% glucose/0.1 mg/ml streptomycin sulphate for 30 min at room temperature. The gel was 'blotted' by gently overlaying it onto an *E. coli* D31 spot assay suspension plate [8], and the plate was incubated at 37 °C for 18 to 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 amino acid composition of purified antibacterial proteins was determined using an automated program on an Applied Biosystems model 420 workstation (Applied Biosystems, Foster City, CA, USA). In samples to be analysed for the presence of tryptophan, tryptophan residues were protected by drying the samples in 20% dodecanethiol in heptane prior to analysis.

The N-terminal amino acid sequence was 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) [15].

The molecular mass of the purified antibacterial proteins was determined by matrix-assisted laser desorption mass spectrometry on a PerSeptive Biosystems Voyager workstation (PerSeptive Biosystems, Framingham, MA, USA).

Antimicrobial assays. The most abundant antibacterial protein was assayed for antibacterial activity using a spectrophotometric assay. Three-millilitre cultures of bacterial strains were grown to mid-logarithmic phase in an appropriate medium [generally trypticase soya broth (TSB)/1% NaCl or Brain Heart Infusion broth, BHI]. The cells were pelleted by centrifugation at 500*g* for 10 min at 4 °C, washed twice in 3 ml of sterile

Table 1. Recovery of antibacterial activity from channel catfish skin extract. One relative unit (U_r) of activity is defined as the ratio of clearing zone diameter in the spot assay of a 4 µl sample to that of 4 µl of cecropin A at 0.2 mg/ml. One absolute unit (U_a) of activity is the amount of activity in 1 ml of sample.

	U_r	Vol (ml)	Total protein (mg)	Specific activity (U_a/µg)	Total activity (U_a)	% Recovery
Extract	0.65	85	194	0.071	13,813	100
HLP-1	0.75	0.2	0.255	0.147	37.5	0.27
HLP-2	0.75	0.1	0.045	0.417	18.75	0.14
HLP-3	0.65	0.1	0.024	0.677	16.25	0.12

phosphate-buffered saline (PBS), and resuspended in PBS to an $OD_{570} = 0.1$ to create a working bacterial stock. Ten microlitres of bacterial stock were added to 1 µl of appropriate control or serially diluted antibacterial protein sample in duplicate sterile microfuge tubes, mixed by repeated pipetting and incubated at 25 °C for 30 min. Following the 30-min incubation, 489 µl of sterile TSB was added to each tube, mixed, and then 100 µl was pipetted into each of 4 wells of a 96-well microtitre plate for each tube. The plate was then incubated at either 25 or 37 °C in a humid environment until control cultures which received only diluent reached an OD_{550} of about 0.080 to 0.120, at which time OD_{550} data was recorded for all samples.

The lethal concentration of the most abundant antibacterial protein was determined using the method of Hultmark et al. [12]. Serial, twofold dilutions of HPLC-purified protein were added to 2-mm diameter wells of an *E. coli* D31 agarose plate. Clearing zone diameters were then measured after incubation at 37 °C for 18 h. Cecropin A (Sigma, St. Louis, MO, USA) was used as a positive control.

Whether the antibacterial activity was due to a cidal or static effect was examined against *E. coli* D31. Small areas of agar were aseptically removed from spot assay plate clearing zones produced by either the catfish antibiotic or magainin 1 (Sigma, St. Louis, MO, USA), aseptically homogenized in 5 ml of TSB/1% NaCl and incubated at 37 °C for 7 days, after which time the OD_{570} was recorded and compared to a sterile media blank to assess microbial growth. Agar was also removed from the bacterial lawn adjacent to the clearing zones as a positive control for bacterial growth.

Antifungal activity was examined by exposing the infective zoospore stage of the water mould *Saprolegnia parasitica* to the antibiotics using a standard exposure assay [16]. Briefly, fungi were propagated in glucose-yeast extract (G-Y) medium [17], and zoospores were produced by placing washed mycelia in sterile sporulation medium (MSM; 0.25 mM $CaCl_2$, 0.25 mM KCl, 20 mM Hepes, 0.01g/l phenol red, pH 7.2). After sporulation, the infective zoospores were collected, and a standard concentration (approx. 10,000 zoospores/ml) was exposed to various treatments for 60 min. All cultures were then diluted fivefold with MSM and reincubated at 20 °C. The number of spores that germinated (i.e. formed germinating hyphae) were then counted using an inverted phase-contrast microscope. The number of germinated spores were again counted after 24 and 48 h to determine the effect of long-term exposure.

Results

The antibacterial activity in acetic acid extracts of channel catfish skin was strongly retained on the cation exchange resin, and eluted at high ionic strength, at about 0.75–1.0 M AA buffer (fig. 2a). Fractions with antibacterial activity were pooled and further fractionated using RP-HPLC, resulting in three major peaks (fig. 2b) each of which had antibacterial activity. The major peak (peak 1), eluting at approximately 50% acetonitrile, was purified to homogeneity (fig. 2c, d) as were the other two proteins as assessed by SDS-PAGE (fig. 3a). The apparent molecular mass of the predominant protein was about 15.5 kD, while the two less

Table 2. Amino acid composition of HLP-1 and trout histone H2B.

Amino acid	# of residues	
	HLP-1	trout H2B*
Asp	6	5
Glu	7	9
Ser	15	14
Gly	18	9
His	3	3
Arg	6	8
Thr	11	9
Ala	19	12
Pro	5	5
Tyr	3	5
Val	9	8
Met	2	2
Ile	6	7
Leu	9	6
Phe	2	2
Lys	14	19

*From ref. 18.

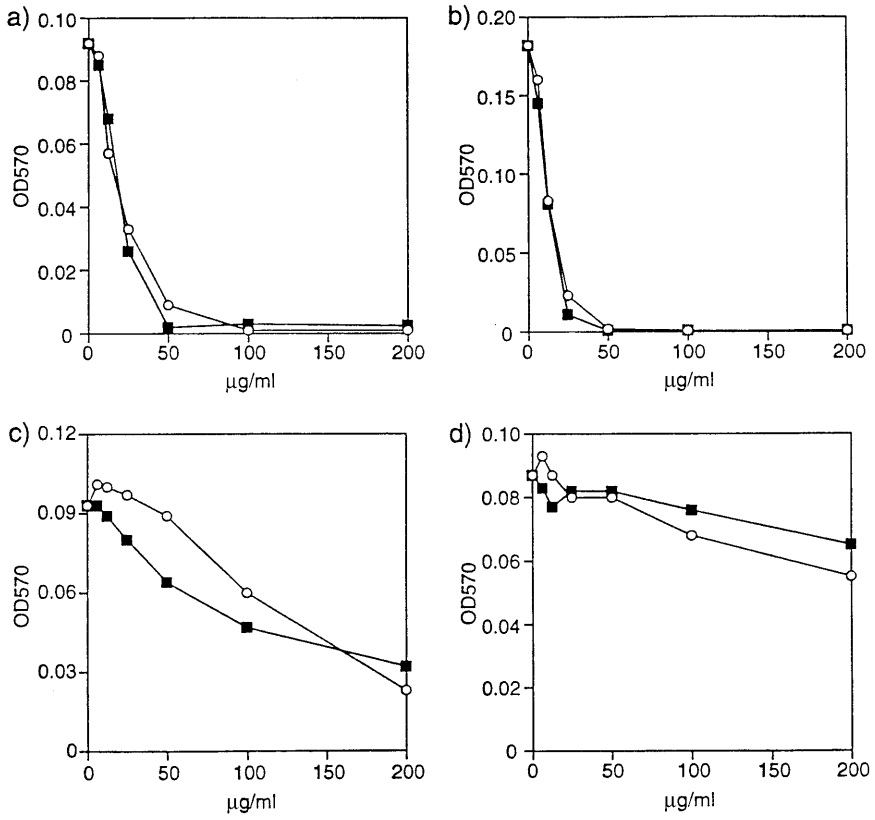

Figure 4. Antibacterial activity of RP-HPLC purified HLP-1 and magainin 1 against various bacterial isolates using the turbidimetric assay. (*a*) *A. hydrophila* 88-5009C2; (*b*) *A. hydrophila* 88-5009C3; (*c*) *V. alginolyticus* 88-5009A2; (*d*) *E. coli* D31. Legend: Closed squares, HLP-1; open circles, magainin 1. Replication of these experiments yielded similar results.

abundant proteins (peaks 2 and 3) were about 30 and 15 kD, respectively (fig. 3a). The yield of the major protein as obtained by RP-HPLC was approximately 15 µg/g skin tissue. Since no assay is yet available to differentiate between the antibacterial proteins we isolated, it was not possible to calculate recovery based on the concentration of each factor in the skin extract. Recovery of each factor based on antibacterial activity was under 0.3%, and the amount of the predominant factor recovered represented about 0.13% of total skin extract protein (table 1).

The SDS-PAGE gel overlay assay revealed a discrete clearing zone corresponding to the position of the major protein (fig. 3b). This indicated that observed antibacterial activity recovered from RP-HPLC peak 1 was due to this protein and not to any factor(s) which may have coeluted.

Mass spectroscopy indicated that the molecular mass of the major protein was 13,459 Da (fig. 2d). Amino acid composition data (table 2) showed that the protein was rich in basic amino acids, especially lysine, and that it lacked tryptophan. A partial N-terminal amino acid sequence yielded PDPAKTAPKKGSKKAVTKXA, which had approximately 89% homology with rainbow trout histone H2B [18]. These two sequences were identical except for the conservative substitutions in the trout sequence of glutamate for aspartate at position 2, and serine for threonine at position 6. We were unable to obtain reproducible mass spectroscopy or sequence data for the protein isolated from RP-HPLC peak 2. However, amino acid composition data indicated that this protein was highly enriched in lysine (data not shown), and had a high lysine-to-arginine ratio, characteristics which are consistent with those of histone H1 [19].

The molecular mass of the least abundant protein isolated from RP-HPLC (peak 3) was 13,506 Da as determined by mass spectroscopy (data not shown), and a partial N-terminal sequence yielded PDPAKTAPKKKSKKAVT. This sequence differed from the major antibacterial protein only by the substitution of lysine for glycine at position 11, and had approximately 82% homology with rainbow trout histone H2B. Due to their similarity to histones, the catfish antibacterial

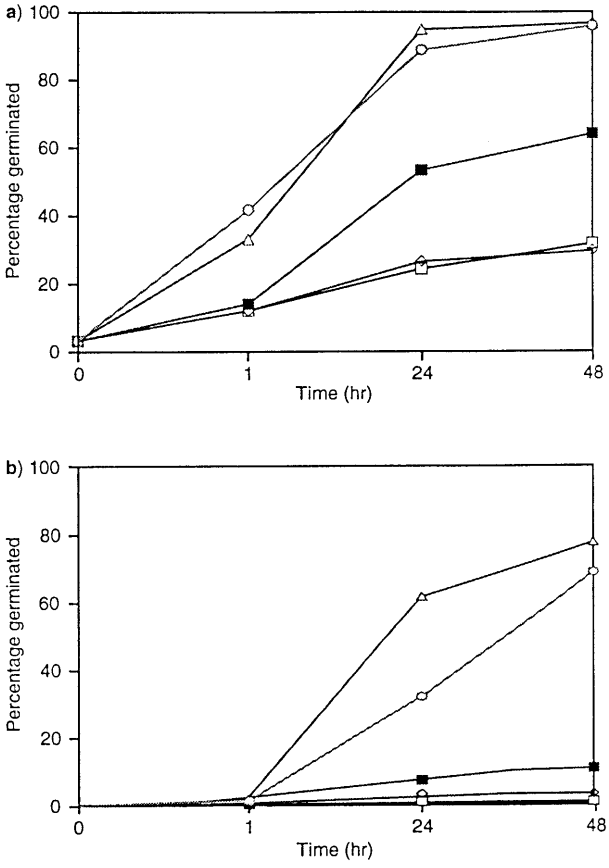

Figure 5. Antifungal activity of HLP-1 and HLP-2 against *Saprolegnia*. (*a*) Isolate from Coho salmon (highly pathogenic to fish [15]); (*b*) Isolate from channel catfish. Quadruplicate wells were inoculated with zoospores (fungal infective stage) having HLP-1 (open squares), HLP-2 (closed squares), magainin 2 (open triangles) or malachite green (closed diamonds). Diluent control (open circles), sham-treated cultures. Replication of these experiments yielded similar results.

proteins we isolated were designated histone-like proteins (HLP) 1, 2 and 3 in decreasing order of predominance in RP-HPLC.

The major protein, HLP-1, had broad-spectrum antimicrobial activity. It was inhibitory to two *Aeromonas hydrophila* isolates and *Vibrio alginolyticus* (fig. 4). Growth of both *A. hydrophila* isolates was almost completely inhibited at as little as 50 μg/ml; magainin 1 was totally inhibitory to *A. hydrophila* 88-5009C3 at 50 μg/ml and completely inhibitory to *A. hydrophila* 88-5009C2 at 100 μg/ml. In contrast, *Staphylococcus aureus* ATCC # 12598 was not inhibited by the catfish antibiotic or magainin 1 (data not shown). Both HLP-1 and magainin 1 were less effective against *A. hydrophila* isolate NCSU 86-8018 than against the other aeromonads tested; *Edwardsiella ictaluri* isolate MSUS94-887 (kindly provided by Dr. Lester Khoo, Mississippi State University) was resistant to both HLP-1 and magainin 1 at concentrations up to 200 μg/ml (data not shown). Both HLP-1 and magainin 1 were cidal to *E. coli* D31 (results not shown). The lethal concentration of HLP-1 against *E. coli* D31 was about 0.37 μM ± 0.023 μM, and that of cecropin A was 0.14 μM, similar to that reported previously [12].

HLP-1, and to a lesser extent HLP-2, possessed significant activity against *Saprolegnia* (fig. 5), which is the most common fungal infection in fish [20, 21]. This antifungal activity was stronger than that of magainin 2, shown to have strong antifungal activity [8], and was even comparable to the clinical dose of malachite green, the most effective chemical used to treat fish for saprolegniosis [22].

Discussion

Channel catfish skin contains three proteins with antimicrobial activity. This activity is acid- and heat-stable, and highly basic in nature, with all antibacterial activity being retained on the CM52 column. We recovered about 15 μg of the predominant antibacterial polypeptide (HLP-1)/g of epithelial tissue. This is a somewhat greater recovery than reported for squalamine from shark mucosal tissues (less than 1 μg/g)[11], but much less than that reported for magainin from *Xenopus* skin (about 2 mg/g) [8]. The total recovery of antibacterial activity from catfish skin extract was only 0.53%, which is similar to the relatively low recovery reported for the cecropins [12]. The recovery of only 0.53% of the activity present in the initial skin extract was less than expected after assuming reasonable loss during purification. The much larger amount of total activity in the extract compared with the purified proteins suggests that the activity of the crude extract may not be proportional to that of the purified HLPs. The activity of the crude extract on spot assay is comparable in relative units to that of the purified HLPs, but the extract had over 180 times as much total activity as the three purified HLPs (table 1). In the absence of additional antibacterial factors in the skin extract, and assuming reasonable loss during purification, recovery of purified activity would be expected to be much higher. The purified activity, however, is greatly concentrated relative to the initial extract, and would not retain detectable antibacterial activity if diluted to the level of the initial extract. As no antimicrobial activity other than the HLPs was detectable, this dramatic difference in total activity recovered may reflect the presence of factor(s) in the crude extract and/or synergism between the HLPs which enhances the antimicrobial activity of the HLPs.

HLP-1 is closely related, if not identical, to histone H2B. It is enriched in the basic amino acid lysine and lacks tryptophan, characteristics which are consistent with those of histone H2B [19]. However, while its amino acid composition varies from that reported for trout H2B (table 2), whether this represents a true difference must await complete sequencing of the molecule.

Although histones have long been known to have antimicrobial properties [23], this report is the first evidence that histones or histone-like proteins may function in fish immunity. However, several reports have recently described antimicrobial functions for histones. Histone H1 may form an antifungal barrier in human epidermis [24]. Histones H2B and H1 have also been isolated from murine macrophages, and have broad-spectrum antibacterial activity against Gram-positive and Gram-negative organisms [25]. A potent, broad-spectrum, antimicrobial peptide termed buforin I, from the stomach of the Asian toad (*Bufo bufo gargarizans*), is a proteolytic cleavage product of histone H2A [26, 27].

Other evidence is suggestive of possible immunological roles for histones as well. Nondividing mouse liver cells and exponentially dividing Friend erythroleukaemia cells contain sizeable cytoplasmic pools of histone H1 [28]. Histones H2B, H2A and H4 have been isolated from bovine milk and serum [29], and N-terminal fragments of histone H2B are found in human wound and blister fluid, along with defensins, lysozyme and the peptide antibiotic FALL-39 [30]. In activated human peripheral blood lymphocytes, histone H2B shifts from an almost exclusively nuclear location to an abundance within the plasma membrane [31]. This plasma membrane H2B binds a sulphated polysaccharide which has been shown to inhibit infection of the cell by human immunodeficiency virus (HIV)-1, further suggesting a possible immunological role for histone H2B. These data suggest a possible significant involvement of at least certain histones in nonspecific immunity.

Several reports also suggest that histones are not confined to the nucleus after synthesis, and may have other roles in addition to their classical involvement in chromatin formation. For example, histone H2A inhibits binding of gonadotropin-releasing hormone [32, 33]. The major constituents of homeostatic thymus hormone are histones H2A and H2B [34]. Heparin-binding proteins from human placenta [35] and human lung carcinoma cell membrane [36] are similar to histone H2B, as is a cytostatic protein isolated from rat foetal lung extracts [37].

Our data suggest that a histone H2B-like protein (HLP-1) is important in the antimicrobial defences of fish skin. The antimicrobial activity which we isolated from catfish skin, of which HLP-1 was the predominant factor, was also present in skin tissue extracts prepared in Tris buffer at physiological pH or in deionized H_2O. These conditions, as well as the mild acid extraction procedure we used, are insufficient to extract histone bound to DNA in the nucleus [38], and thus these factors are readily available to participate in host defence.

The broad-spectrum antimicrobial activity of HLP-1 also supports the hypothesis that this factor is involved in innate immunity. This protein is inhibitory to fish bacterial pathogens. The lethal concentration of HLP-1 against *E. coli* D31 is also comparable to the potent insect defence peptides, the cecropins [11]. Additionally, both HLP-1 and HLP-2 possess strong antifungal activity against *Saprolegnia*, the most important fungal pathogen of fish. Taken together, our data suggest that several histone-like proteins in fish may be important as a first line of defence against microbial colonization.

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1 Ellis A. (1982) Differences between the immune mechanisms of fish and higher vertebrates. In: Microbial Diseases of Fish, pp. 1–30, Roberts R. (ed.), Academic Press, London
2 Avtalion R., Weiss E. and Moalem T. (1976) Regulatory effects of temperature upon immunity in ectothermic vertebrates. In: Comparative Immunology, pp. 227–238, Marchalonis J. (ed.), Blackwell, Oxford
3 Bly J. and Clem W. (1991) Temperature-mediated processes in teleost immunity: in vitro immunosuppression induced by in vivo low temperature in channel catfish. Vet. Immunol. Immunopathol. **28:** 365–377
4 Roberts R. (1989) Fish Pathology, 2nd ed., Bailliere Tindall, London
5 Boman H. (1995) Peptide antibiotics and their role in innate immunity. Ann. Rev. Immunol. **13:** 61–92
6 Selsted M., Brown D., DeLange R. and Lehrer R. (1983) Primary Structures of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages. J. Biol. Chem. **258:** 14485–14489
7 Lehrer R., Lichtenstein A. and Ganz T. (1993) Defensins – antimicrobial and cytotoxic peptides of mammalian cells. Ann. Rev. Immunol. **11:** 105–128
8 Zasloff M. (1987) Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. USA **84:** 5449–5453
9 Steiner H., Hultmark D., Engstrom A., Bennich H. and Boman H. (1981) Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature **292:** 246–248
10 Lemaitre C., Orange N., Saglio P., Saint N., Gagnon J. and Molle G. (1996) Characterization and ion channel activities of

novel antibacterial proteins from the skin mucosa of carp (*Cyprinus carpio*). Eur. J. Biochem. **240:** 143–149
11 Moore K., Wehrli S., Roder H., Rogers M., Forrest J., Mccrimmon D. et al. (1993) Squalamine – an aminosterol antibiotic from the shark. Proc. Natl. Acad. Sci. USA **90:** 1354–1358
12 Hultmark D., Engstrom A., Bennich H., Kapur R. and Boman H. (1982) Insect immunity: isolation and structure of Cecropin D and four minor antibacterial components from cecropia pupae. Eur. J. Biochem. **127:** 207–217
13 Laemmli U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685
14 Blank A., Silber J., Thelen M. and Dekker C. (1983) Detection of enzymatic activities in sodium dodecyl sulfate-polyacrylamide gels: DNA polymerases as model enzymes. Anal. Biochem. **135:** 423–430
15 Altschul S., Gish W., Miller W., Myers E. and Lipman D. (1990) Basic local alignment search tool. J. Molec. Biol. **215:** 403–410
16 Yuasa K. and Hatai K. (1995) Drug sensitivity of some pathogenic water moulds isolated from freshwater fishes. Journal of Antibacterial and Antifungal Agents **23:** 213–219
17 Hatai K. and Egusa S. (1979) Study on the pathogenic fungus of mycotic granulomatosis III. Development of the medium for MG fungus. Fish Pathol. **13:** 147–152
18 Kootstra A. and Bailey G. (1978) Primary structure of histone H2B from trout (*Salmo trutta*) testes. Biochemistry **17:** 2504–2510
19 Isenberg I. (1979) Histones. Ann. Rev. Biochem. **48:** 159–192
20 Neish G. and Hughes G. (1980) Fungal Diseases of Fishes, T.F.H. Publications, Neptune City, NJ
21 Noga E. (1993) Fungal diseases of freshwater fishes: recent advances. Ann. Rev. Fish Diseases **3:** 291–304
22 Noga E. (1996) Fish Disease: Diagnosis and Treatment, Mosby-Year Book, St. Louis, MO
23 Hirsch J. (1958) Bactericidal action of histone. J. Exp. Med. **108:** 925–944
24 Kashima M. (1991) H1 histones contribute to candidacidal activities of human epidermal extract. J. Dermatol. **18:** 695–706
25 Hiemstra P., Eisenhauer P., Harwig S., Van Den Barselaar M., Van Furth R. and Lehrer R. (1993) Antimicrobial proteins of murine macrophages. Infect. Immun. **61:** 3038–3046
26 Park C., Kim M. and Kim S. (1996) A novel antimicrobial peptide from *Bufo bufo gargarizans*. Biochem. Biophys. Res. Commun. **218:** 408–413
27 Kim H., Park C., Kim M. and Kim S. (1996) cDNA cloning and characterization of buforin I, an antimicrobial peptide: a cleavage product of histone H2A. Biochem. Biophys. Res. Commun. **229:** 381–387
28 Zlatanova J., Srebreva L., Banchev T., Tasheva B. and Tsanev R. (1990) Cytoplasmic pool of histone H1 in mammalian cells. J. Cell Sci. **96:** 461–468
29 Waga S., Tan E. and Rubin R. (1987) Identification and isolation of soluble histones from bovine milk and serum. Biochem. J. **244:** 675–682
30 Frohm M., Gunne H., Bergman A., Agerberth B., Bergman T., Boman A. et al. (1996) Biochemical and antibacterial analysis of human wound and blister fluid. Eur. J. Biochem. **237:** 86–92
31 Watson K., Edwards R., Shaunak S., Parmelee D., Sarraf C., Gooderham N. et al. (1995) Extra-nuclear location of histones in activated human peripheral blood lymphocytes and cultured T-cells. Biochem. Pharmacol. **50:** 299–309
32 Aten R. and Behrman H. (1989) A gonadotropin-releasing hormone-binding inhibitor from bovine ovaries. Purification and identification of histone H2A. J. Biol. Chem. **264:** 11065–11071
33 Aten R. and Behrman H. (1989) Antigonadotropic effects of bovine ovarian gonadotropin-releasing hormone-binding inhibitor/histone H2A in rat luteal and granulosal cells. J. Biol. Chem. **264:** 11072–11075
34 Reichhart R., Zeppezauer M. and Jornvall H. (1985) Preparations of homeostatic thymus hormone consist predominately of histones 2A and 2B and suggest additional histone functions. Proc. Natl. Acad. Sci. USA **82:** 4871–4875
35 Kohnke-Godt B. and Gabius H. (1991) Heparin-binding lectin from human placenta: further characterization of ligand binding and structural properties and its relationship to histones and heparin-binding growth factors. Biochemistry **30:** 55–65
36 Bilozur M. and Biswas C. (1990) Identification and characterization of heparin sulfate-binding proteins from human lung carcinoma cells. J. Biol. Chem. **265:** 19697–19703
37 Conliffe P., Bennett H. and Mulay S. (1993) Isolation and characterization of a cytostatic histone H2B-like protein from fetal lungs of non-diabetic and diabetic rats. J. Endocrinol. **139:** 97–105
38 Johns E. (1977) The isolation and purification of histones. Methods Cell Biol. **16:** 183–203