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Purification of antimicrobial factor from granules of channel catfish peripheral blood leucocytes

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Abstract

The channel catfish (*Ictalurus punctatus*) is extensively used in aquaculture in the Southeast US and is susceptible to many bacterial infections acquired from its pond environment. Research is needed to better understand the defensive response and innate immunity of channel catfish against fish pathogens like *Edwardsiella ictaluri* and *Aeromonas hydrophila*. The main objectives were purification and characterization of an innate antimicrobial factor isolated from catfish leucocytes that has both bactericidal and antiviral activities. Oxygen-independent mechanisms of innate immunity for killing microorganisms have not been identified in leucocytes of channel catfish. Leucocytes were separated from catfish blood, and granule extracts were obtained by homogenization, centrifugation, and extraction with 10% acetic acid. The granule extracts were further purified by gel filtration chromatography. Bactericidal assays against the two fish pathogens and SDS–PAGE analysis were done on the isolated antimicrobial factor. Determination of antiviral activity of the factor was done by in vitro tissue culture using herpes simplex virus-type 1. Mass spectrometry analyses were done for molecular weight (655 Da), purity, and structural characterization of the innate non-peptide antimicrobial factor.

Keywords: Antimicrobial factor; Purification; Bactericidal; Channel catfish

The channel catfish (*Ictalurus punctatus*) is extensively used in aquaculture or fish farming in the Southeast US. The channel catfish is susceptible to many bacterial infections acquired from its pond environment. Enteric septicemia of catfish (ESC), caused by *Edwardsiella ictaluri*, is a major bacterial disease of channel catfish in pond culture and causes high mortality [1–3]. Further research is needed then to understand catfish innate immunity against *E. ictaluri* and *Aeromonas hydrophila* which are important fish pathogens.

The main objective of the research was purification by gel filtration chromatography of an antimicrobial factor from catfish leucocyte granules. Determination of the molecular weight, purity, and structural characterization of the antimicrobial factor, its bactericidal activity against two important fish pathogens, *E. ictaluri* and *A. hydrophila*, and determination of its antiviral activity against herpes simplex virus-type 1 (HSV-1) were done.

We demonstrated that an antimicrobial factor exists in teleost fish leucocytes that kills catfish bacterial and viral pathogens. The importance of these findings is that channel catfish peripheral blood leucocytes are capable of killing fish pathogens such as *E. ictaluri*, *A. hydrophila*, and herpesviruses [4–6]. Antimicrobial compounds important in innate immunity [7] have not been identified in leucocytes of channel catfish [7–9]. At least 10 antimicrobial peptides and proteins have been identified in mammalian leucocytes, especially neutrophils [10].

This investigation provided new information about the defensive response of catfish leucocytes to bacterial pathogens and viruses and expanded our previous research on catfish innate immunity via the alternative complement pathway [11,12]. The antimicrobial factor found here in granules of catfish leucocytes was purified and characterized with regard to its molecular weight,

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structure, and antimicrobial activities. Pursuit of this and other antimicrobial factors may lead to development of new compounds for the treatment of microbial infections.

Materials and methods

Maintenance of channel catfish. Adult catfish were maintained in tanks at 27 °C in a recirculating water-reuse culture system at the Ecological Research Center (Dr. Bill Simco, Director), Department of Biology, University of Memphis [11–13]. Channel catfish were provided by Dr. William Wolters, USDA-ARS, Catfish Genetics Research Unit, Stoneville, MS.

Separation of leucocytes from channel catfish blood. Fifty milliliters of heparinized blood was obtained by bleeding from the caudal sinus 2-year-old catfish that had been anesthetized with tricaine methanesulfonate [11–13]. The blood was diluted 1:2 with calcium–magnesium–free Hanks' balanced salt solution (CMF-HBSS), pH 7.2 [5,14]. Five milliliters of 1:2 CMF-HBSS diluted blood was layered over the top of 3 ml of Histopaque 1077 (Sigma, St. Louis, MO) using 16 × 125 mm (15 ml) conical centrifuge tubes [5,14]. Centrifugation at 300*g* for 30 min at room temperature (25 °C) was done. After centrifugation and washing of the white blood cells two times in phosphate-buffered saline, pH 7.2, leucocytes were obtained from the opaque interface of the density gradient. Using the 1077 Histopaque (Sigma) for cell separation of channel catfish peripheral blood leucocytes, Waterstrat et al. [14] obtained leucocyte counts for monocytes (6.5%), neutrophils (14.6%), lymphocytes (27.4%), and thrombocytes/small lymphocytes (50.9%).

Isolation of antimicrobial factor from channel catfish leucocytes. Leucocytes were isolated from catfish peripheral blood using the Histopaque 1077 gradient (Sigma) as described above. The leucocytes were resuspended in 1 ml of 0.34 M sucrose, 0.02 M potassium phosphate homogenizing buffer, pH 7.4 [15]. The leucocytes were homogenized on ice using a pellet pestle motor (Kontes, Vineland, NJ) until the cells appeared broken by phase-contrast microscopy. The cell debris and unbroken cells were removed by low-speed centrifugation at 200*g* for 10 min and the granule-rich supernatant saved. Three more cycles of homogenization in the homogenizing buffer were done, using each time the residual cell pellet and centrifugation at low speed to obtain additional granule-rich supernatants. All of the supernatants were combined and centrifuged at 27,000*g* for 30 min at 4 °C. The resulting pellets were pooled and extracted overnight in 10% acetic acid at 4 °C. The extract was centrifuged at 27,000*g* for 30 min and the supernatant saved. The supernatant was concentrated using a Speed-Vac vacuum concentrator (Savant, Farmingdale, NY).

Column chromatography and purification of antimicrobial factor. Gel filtration chromatography was done on the leucocyte granule extracts using a Bio-Gel P-10 (Bio-Rad, Richmond, CA) column (1 × 18 cm) and elution with 0.1 M sodium phosphate buffer, pH 7.4 [15]. As much as 0.2 ml of extract (247 μg/ml protein) was applied to the column with 0.5 ml of eluate collected per tube. Ultraviolet absorption was monitored at 280 nm. The molecular weight fractionation range of the P-10 gel is 1.5–20 kDa. Bactericidal assays were done on fractions of the peaks obtained by gel filtration using *Escherichia coli*, *E. ictaluri*, and *A. hydrophila*. Molecular weight determination, SDS–PAGE, and mass spectrometry were performed on the purified antimicrobial factor.

Bactericidal assays. Two bactericidal assays (tube assay and well assay) were done on the purified catfish bactericidal factor. Assay determinations were done in triplicate.

To conduct the bactericidal tube assay, a bacterial species of *E. ictaluri* (ATCC 33202), *A. hydrophila* (ATCC 7966), and *Escherichia coli* (K12 D31 strain; obtained from Dr. Hans Boman, University of Stockholm, Sweden) were grown in tryptic soy broth or brain heart infusion broth for 24 h at 30–35 °C (depending on the species). The bactericidal assay was done as previously described using 50 μl of a culture dilution of bacteria in saline and 50 μl of the catfish bactericidal factor or 50 μl of sterile saline [11–13,16,17]. The incubation time of the mixture was 90 min at 30 °C. The mixture was added to 10 ml of molten tryptic soy agar (TSA) or brain heart infusion agar (BHA) in a petri plate and swirled to mix. The TSA or BHA petri plates were incubated for 48 h at 30–35 °C (depending on the species) and the bacterial colonies were then counted. An equation was used to calculate the percent bactericidal activity of the catfish bactericidal factor [11–13].

Bactericidal activity of the leucocyte bactericidal factor was also determined using a well diffusion assay [16]. Petri plates containing 10 ml of Luria 1% agarose, pH 6.4, were prepared. The Luria agarose contained 0.1 M sodium phosphate and 2.5×10^5 cells of *E. coli* K12 D31. Ten microliters of the purified antimicrobial factor obtained from Bio-Gel P-10 chromatography was added to wells cut 3 mm in diameter. After 24–48 h, the diameter of the zone of inhibition of growth of *E. coli* K12 D31 was measured in millimeters using a Kallestad (Chaska, MN) calibrated viewer.

To conduct the well diffusion assay for bactericidal activity against *A. hydrophila*, petri plates containing 10 ml of tryptic soy agarose, pH 7.3, and 2.5×10^5 bacteria were used. Wells of 3 mm in diameter were cut and filled with 10 μl of the appropriate antimicrobial factor preparation and then observed and measured for inhibition of growth as described above.

To conduct the well diffusion assay for bactericidal activity against *E. ictaluri*, petri plates containing 10 ml of brain heart infusion agarose, pH 7.4, and 2.5×10^5 bacteria were used. Wells of 3 mm in diameter were cut and filled with 10 μl of the appropriate antimicrobial factor preparation and then observed and measured for inhibition of growth as described above.

To conduct the well diffusion assay for lysozyme activity against *Micrococcus lysodeikticus*, petri plates containing 10 ml of tryptic soy agarose, pH 7.3, were mixed with 60 μl of a 10^{-2} dilution of *M. lysodeikticus* [18,19]. Wells of 3 mm in diameter were cut and filled with 10 μl each of tube fractions 9, 10, 11, and 12 from Bio-Gel P-10 chromatography (Fig. 3). The plates were incubated for 24–48 h at 37 °C and the zones of inhibition of growth were measured as described above.

Antiviral assay against herpes simplex virus-1. The isolated antimicrobial factor from the catfish granule extract was tested for its antiviral activity against HSV-1 in tissue culture. Antiviral activity was determined using a quantitative assay done in gingival fibroblast cell tissue culture in DMEM that demonstrated viral cytopathic effect (CPE) and plaque formation [17,20,21]. The antiviral factor was added to the cell monolayer 24 h before infection with HSV-1. These results were compared with those of phosphate-buffered saline, pH 7.2, being added to the tissue culture instead of the antiviral factor before infection with HSV-1. Three days of incubation at 37 °C were done before reading the CPE results.

SDS–PAGE analysis of antimicrobial factor. Molecular weight and general analysis of the antimicrobial factor and any other granular proteins, peptides or compounds were determined using an SDS–polyacrylamide gel electrophoresis method for low molecular weight proteins (modification of the discontinuous procedure of Schagger and von Jagow [22]; Sigma Technical Bulletin MWM-100). The SDS gel had a separating gel of 16.5% acrylamide with a 10% acrylamide spacer gel and 4% stacking gel. The gel (8 × 10 cm and 0.75 mm thick) was subjected to electrophoresis for 2 h at 90 mA. The gel was then stained for protein with Coomassie blue and by silver staining. Low molecular weight standards (Bio-Rad, Hercules, CA) used were phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), hen egg ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), hen egg lysozyme (14.4 kDa), and aprotinin (6.5 kDa). Myoglobin digest standards (Pharmacia, Piscataway, NJ) consisted of myoglobin (17 kDa), myoglobin I and II (14 kDa), myoglobin I (8.1 kDa), myoglobin II (6.2 kDa), and myoglobin III (2.5 kDa).

Protein assay. Protein concentration of the channel catfish antimicrobial factor was determined using the Bradford [23] protein assay (Bio-Rad, Hercules, CA). Bovine serum albumin was used as the protein standard.

Amino acid sequence analysis. An amino acid sequence analysis of the antimicrobial factor was done by sequential Edman degradation using a gas phase automatic sequencer (Applied Biosystems, Foster City, CA, Model 477A) equipped with an online automatic amino acid analyzer (ABI Model 420). The amino acid sequence analysis was done at the Center for Biotechnology, St. Jude Children's Research Hospital, Memphis, TN.

Mass spectrometry. The molecular weight, purity, and structural characterization of the isolated antimicrobial factor were determined using mass spectrometric methods [24,25] using a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI-TOF-MS) instrument (Voyager DERP, Perseptive Biosystems, Framingham, MA). Electrospray ionization-MS/MS was done using a ThermoQuest Finnigan LCQ DECA (ThermoFinnigan, San Jose, CA) instrument. The MALDI-TOF-MS and electrospray ionization-MS/MS analyses were done at the Charles B. Stout Mass Spectrometry Laboratory, University of Tennessee Health Science Center, Memphis, TN.

Nuclear magnetic resonance. Nuclear magnetic resonance (NMR) structural analysis of the antimicrobial factor was performed on a Varian high-field 600 MHz NMR spectrometer with Oxford magnet (Varian Analytical Instruments, Valencia, CA; Oxford Instruments, Fremont, CA). The NMR spectrometer has a 14.0 T field strength magnet (Oxford), a ^{1}H NMR frequency of 600 MHz, and a ^{13}C NMR frequency of 125.7 MHz. Multinuclear capabilities allow the observation of most NMR active nuclei. NMR structural analysis of the antimicrobial factor was done by the Department of Structural Biology, St. Jude Children's Research Hospital, Memphis, TN.

Results

A blood cell fraction of leucocytes was isolated from channel catfish peripheral blood by density gradient centrifugation. A procedure that specifically extracts a granular-rich sediment from human neutrophils was used for the catfish leucocytes [15]. The leucocytes were first homogenized to obtain granule-rich supernatants by low-speed centrifugation. The supernatants were then combined and subjected to high-speed centrifugation to obtain a pellet of granules. A 10% acetic acid extract of the pellet granule was then made, concentrated by Speed-Vac, and reconstituted in water. The granule extract, by the tube assay, showed 100% bactericidal activity against *E. coli* K12 D31 and 100% bactericidal activity against both *E. ictaluri* and *A. hydrophila* (Table 1), both well-known pathogens of channel catfish in pond culture. The granular extract, by the well assay using Luria agarose inoculated with *E. coli* K12 D31, gave a well diameter of clearing of 10 mm after 48 h (Table 1; Fig. 1A). The granular extract, again done by the well assay, gave a diameter of clearing of 12.1 mm by inoculation of agarose gel with *E. ictaluri* and 14.7 mm of clearing with *A. hydrophila* (Table 1; Fig. 1B). The diameter of the well was 3 mm, so these are certainly satisfactory results that demonstrated bactericidal activity by the antimicrobial factor. Most of the antibacterial activity against *E. coli* is found in the azurophil granule of mammalian neutrophils and is associated with a pelletable material upon granule disruption [26].

The 10% acetic acid granular extract (247 μg/ml protein) was diluted in 0.1 M sodium phosphate buffer,

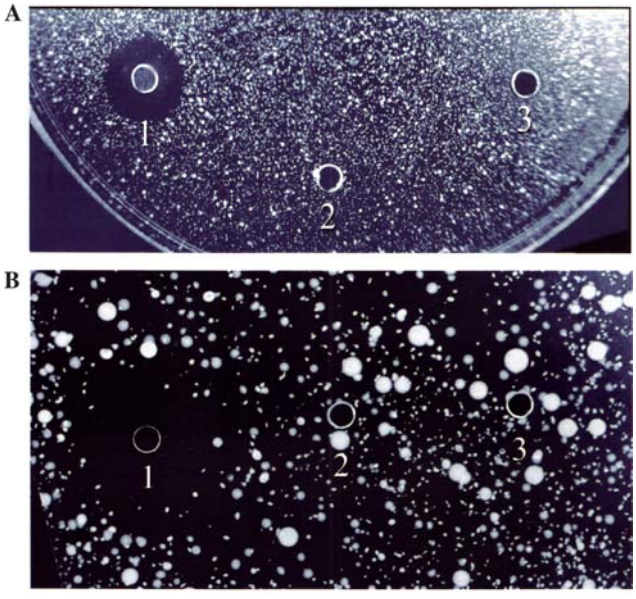

Fig. 1. (A) Well diffusion assay with *Escherichia coli* K12 D31 in 1% Luria agarose, pH 6.4, at 48 h. Well 1: 10% acetic acid extract in 0.1 M sodium phosphate buffer, pH 7.4; well 2: cell lysate supernatant; well 3: 10% acetic acid. (B) Well diffusion assay with *Aeromonas hydrophila* in tryptic soy agarose, pH 7.3, at 48 h. Well 1: 10% acetic acid extract in 0.1 M sodium phosphate buffer, pH 7.4; well 2: cell lysate supernatant; well 3: 10% acetic acid.

Table 1
Bactericidal activity (well diffusion and tube assays) of granular extract obtained from channel catfish peripheral blood leucocytes

Preparation[a]	*Escherichia coli*		*Edwardsiella ictaluri*		*Aeromonas hydrophila*	
	Well[b]	Tube (%)[c]	Well[b]	Tube (%)[c]	Well[b]	Tube (%)[c]
10% acetic acid extract	10 mm	100	12.1 mm	100	14.7 mm	100
Bio-Gel P-10 filtration (Tube #16)	ND	29	ND	37	ND	43
Bio-Gel P-10 filtration (Tube #17)	ND	31	ND	38	ND	46

ND, not done.
[a] Preparations in 0.1 M sodium phosphate buffer, pH 7.4.
[b] Zone of inhibition (diameter in mm) at 48 h.
[c] Percent bactericidal activity at 48 h.

pH 7.4, to determine the minimum inhibitory concentration of the antimicrobial factor against *E. ictaluri* in BHA, pH 7.4 (Table 2). An eightfold dilution still gave 78% bactericidal activity, with 19% bactericidal activity being found at a 32-fold dilution (7.7 μg/ml protein) which was the last dilution tested. This shows that the bactericidal activity was concentration-dependent and could be diluted out, with the percent bactericidal activity decreasing by about one-half with each of the higher dilutions of 1:8, 1:16, and 1:32 (Table 2). This indicates then the least amount of antimicrobial factor that was still bactericidal, and that the antimicrobial factor was homogeneous since its bactericidal activity was concentration-dependent.

Table 2
Percent bactericidal activity of dilutions in 0.1 M sodium phosphate buffer, pH 7.4, of 10% acetic acid granular extract* versus *Edwardsiella ictaluri*

Dilution	Percent bactericidal activity (%)
1:2	100
1:4	100
1:8	78
1:16	40.5
1:32	18.5

The assay was done in brain heart infusion agar to determine the minimum inhibitory concentration of the antimicrobial factor.
* 247 μg/ml protein.

By SDS–PAGE using a 4–10–16.5% discontinuous gradient gel [18,22,27,28], the 10% acetic acid extract of the granular pellet gave 10 distinct bands by silver staining (Fig. 2A). The bands were found throughout a molecular weight range of 6.5–97.4 kDa using standard protein markers. The granular extract therefore contained many different proteins. No protein bands of less than 6.5 kDa (the aprotinin standard) were seen by SDS–PAGE of the 10% acetic acid granular extract (Fig. 2A). This indicates that natural antibiotic peptides, like the defensins (4 kDa) found in human neutrophil azurophil granules [15,29], were absent in our catfish peripheral white blood cell preparation (Figs. 2A and B).

The granule extract was applied to a Bio-Gel P-10 column (molecular weight fractionation range of 1.5–20 kDa) and two peaks were obtained after monitoring at 280 nm (Fig. 3). The 10% acetic acid extract (0.2 ml) applied to the Bio-Gel P-10 gel column had 49.4 μg total protein by the Bio-Rad protein assay [23]. All of the tubes from column chromatography fractionation were sterile filtered and assayed for their percent bactericidal activity against *E. coli* K12 D31. The greatest

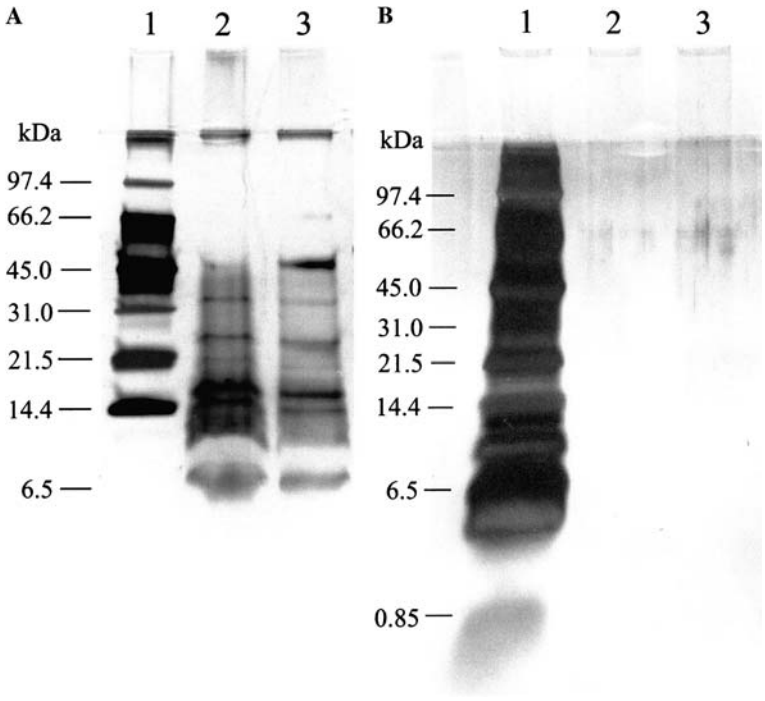

Fig. 2. (A) SDS gel electrophoresis of 10% acetic acid granular extract done under non-reducing and reducing conditions. The gel was visualized by silver staining. No stained bands were seen in the low molecular weight region of the gel. Lane 1: standard proteins (Bio-Rad); lane 2: granular extract done under non-reducing condition; lane 3: granular extract done under reducing condition (2-ME reduction). Molecular weight standards in kilodaltons (kDa) are shown on left. (B) SDS gel electrophoresis of tube fractions 16 and 17 from Bio-Gel P-10 gel filtration. Tube fractions 16 and 17 contained the greatest bactericidal activity. The gel was visualized by first staining with Coomassie blue and then silver stained. No stained bands were seen in the low molecular weight region of the gel. Lane 1: standard proteins (Bio-Rad) and Coomassie brilliant blue G tracking dye, 854 Da; lane 2: tube number 16; and lane 3: tube number 17. Molecular weight standards in kilodaltons (kDa) are shown on the left.

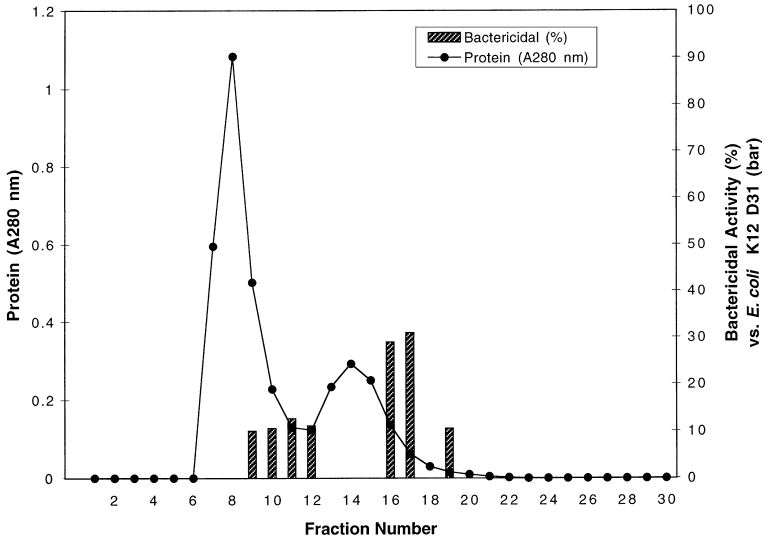

Fig. 3. The channel catfish leucocyte granular extract (0.2 ml; 247 μg/ml protein) was applied to a Bio-Gel P-10 column (1 × 18 cm) in 0.1 M sodium phosphate buffer, pH 7.4, and monitored at 280 nm. Elucate (0.5ml) was collected per tube. Tube fractions 16 and 17 contained the greatest bactericidal activity against the three bacterial genera and antiviral activity against herpes simplex virus-type1. A molecular weight standard (Vitamin B-12, 1.35 kDa), applied to the same Bio-Gel P-10 column, eluted at tube number 15 at OD 280 nm.

bactericidal activities (29% and 31%) against *E. coli* K12 D31 were found, respectively, in the two tube fractions 16 and 17 eluted at the end of the second peak after Bio-Gel P-10 filtration (Table 1; Fig. 3). Both of these tube fractions had a molecular weight less than the eluted 1.35 kDa vitamin B-12 standard. Tube number 17 also showed bactericidal activity of 38% against *E. ictaluri* and 46% against *A. hydrophila* (Table 1). No protein was detected by the Bio-Rad protein assay in tubes 16 and 17.

When both tube fractions 16 and 17 showing the greatest bactericidal activity from Bio-Gel P-10 isolation (Fig. 3) were analyzed on an SDS–PAGE low molecular weight gel [22], no bands were seen after silver staining for protein (Fig. 2B). This indicates that the antimicrobial factor is not a peptide and that no other higher molecular weight peptides or proteins were present. Since small peptides below 1000 Da do not stain well, this result does not completely rule out a peptide as the antimicrobial factor, nor does it rule out amino acid derivatives [10,29]. However, no amino acid sequence was detected when the antimicrobial factor was subjected to Edman degradation. The N-terminal end though could possibly be blocked, in which case no amino acid sequence would be detected. Electrospray ionization-MS/MS was then done which also failed to detect amino acids and therefore concluded that the antimicrobial factor was not a peptide. An elemental analysis of the percent of weight of sample (Desert Analytics, Tucson, AZ) found the elements carbon (31.4%), hydrogen (6.6%), and nitrogen (<0.05%) present in the antimicrobial factor. Carbon and hydrogen were present at about a 1:2 molar ratio. Nitrogen was too low in percentage to say if the element is or is not present.

The antimicrobial factor then is not a peptide for the following reasons. No protein was detected by the Bio-Rad protein assay in tubes 16 and 17 from Bio-Gel P-10 gel filtration, both tubes of which though showed the greatest bactericidal activity (Table 1; Fig. 3). No protein bands were seen by low molecular weight-SDS–PAGE of tubes 16 and 17 after silver staining (Fig. 2B). Also, no protein bands were seen in the low molecular weight region of the 10% acetic acid extract done by SDS–PAGE under both reducing and non-reducing conditions and then silver stained (Fig. 2A). No amino acid sequence could be detected for the antimicrobial factor by Edman degradation or by electrospray ionization-MS/MS. Furthermore, the factor contained almost no nitrogen by elemental analysis.

A MALDI-TOF-MS analysis was done on the two tube fractions 16 and 17 that had the greatest bactericidal activity from Bio-Gel P-10 fractionation of the granule extract. The MALDI-TOF-MS analysis showed purity of both tubes and contained a compound having a molecular weight of 655 Da (656 Da-1 proton) (Fig. 4).

A pool of the two tube fractions 16 and 17, containing the greatest bactericidal activity from Bio-Gel P-10 fractionation of the granular extract, was assayed for antiviral activity against HSV-1. A 60% reduction in the cytopathic effect (CPE), or the visible effect that viruses have on tissue culture cells, was seen when gingival fibroblast cells were treated with the purified granule extract fraction versus the untreated phosphate-buffered saline, pH 7.2, control. A 30% CPE was seen in the 3-day test with the catfish leucocyte granular extract

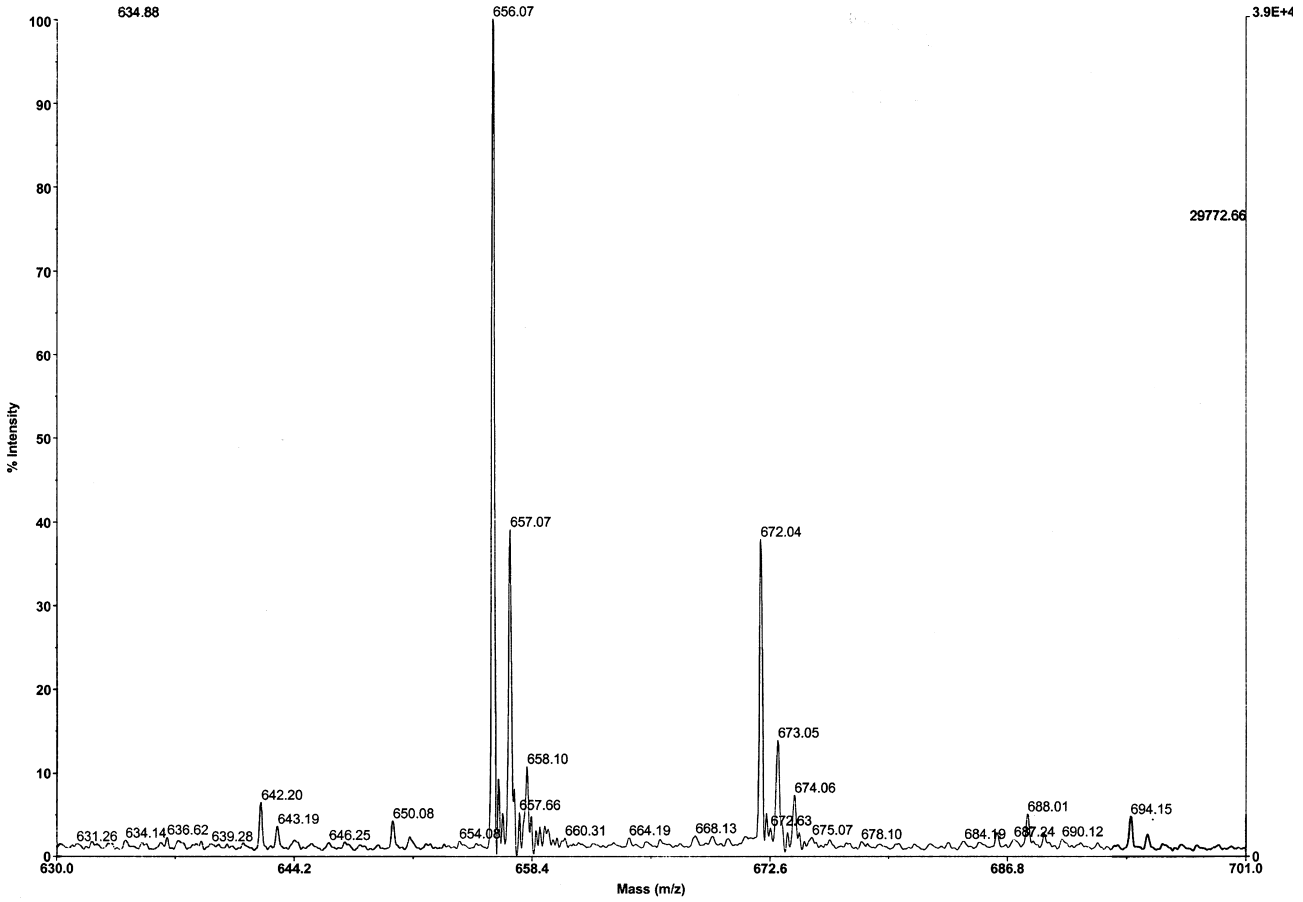

Fig. 4. Molecular weight determination of antimicrobial factor isolated from channel catfish peripheral blood leucocytes. MALDI-TOF-mass spectrometry determined the molecular weight of tube fractions 16 and 17 to be 655 Da (656 Da-1 proton).

versus 90% CPE for the phosphate buffered saline, pH 7.2-treated gingival fibroblast cells after their infection with HSV-1. The antibacterial factor therefore also has antiviral activity against HSV-1 in in vitro tissue culture.

The low bactericidal activity against *E. coli* K12 D31 (10–12% range) observed in tube fractions 9, 10, 11, and 12 from Bio-Gel P-10 fractionation could possibly represent lysozyme activity (Figs. 2A and 3). These four tube fractions are within the molecular weight range of about 14 kDa for lysozyme (Fig. 3). Lysozyme was previously found and quantitated in channel catfish skin mucus [30] and serum [13]. The channel catfish leucocyte granular extract as prepared here could contain lysozyme, and this possibility was tested for by a well diffusion assay of the four tube fractions 9–12 with *M. lysodeikticus* [18,19]. No lysozyme activity could be detected.

Discussion

We have extensively investigated immunity in the channel catfish concentrating on the complement system [5,11–13,28,30–35]. It was found that both the classical and alternative complement pathways are activated and can have a bactericidal effect against catfish pathogens. Bactericidal activity by catfish serum, mediated by the alternative complement pathway, was an important means of defense against many Gram-negative bacteria. The alternative complement pathway is an aspect of innate immunity in channel catfish. The presence and amount of sialic acid found on the surface of Gram-negative bacteria determined the effectiveness of the alternative complement pathway in killing the bacteria tested including all of the major bacterial pathogens of channel catfish. Bactericidal activity via the alternative complement pathway was greatest against bacteria lacking sialic acid on their surfaces and least effective against those bacteria that had large amounts of surface sialic acid, which included *E. ictaluri* and all of the other major bacterial pathogens of channel catfish. The alternative complement pathway was found to be important in opsonization of bacteria for enhancing phagocytosis. The age of catfish was also found to be a factor in the effectiveness of the alternative complement pathway and bactericidal activity, as bactericidal activity increased with age against *Pseudomonas fluorescens*. However, no bactericidal activity by the alternative

complement pathway was found against *E. ictaluri* or *A. hydrophila* at any age.

In mammals, neutrophils and macrophages destroy microorganisms in phagocytic vacuoles by oxygen-dependent and oxygen-independent mechanisms [36]. Fish phagocytes are known to carry out the respiratory burst that generates reactive oxygen species [8]. This is an oxygen-dependent system that is apparently bactericidal in fish [8]. In the oxygen-independent system, at least 10 major antimicrobial proteins have been identified in acid extracts from granules of mammalian neutrophils [26]. Bactericidal proteins like the serprocidins (25–29 kDa) and defensins (4 kDa) represent oxygen-independent mechanisms of immunity used by mammalian phagocytes for intracellular killing of microorganisms [26,29,37,38]. Oxygen-independent antimicrobial factors have not been identified in catfish leucocytes until this study.

Antimicrobial peptides and proteins play important roles in innate immunity and are widespread throughout the animal kingdom. We previously identified lysozyme and IgM in the skin mucus of the channel catfish [30]. A 25-residue antimicrobial peptide was identified in the skin mucus of the winter flounder that has broad-spectrum bactericidal activity [39]. Douglas et al. [40] found genomic sequences of pleurocidin-like antimicrobial peptides present in skin and intestinal cells of winter flounder. Robinette et al. [41] have found a histone-like protein in channel catfish skin that has antibacterial activity. Richards et al. [42] isolated a histone H1-type antimicrobial protein from Atlantic salmon liver extracts. Cho et al. [43] found a parasin I antimicrobial peptide in *Parasilurus* catfish skin mucosa that was derived from histone H2A. Silphaduang and Noga [44] isolated piscidin-like peptide antibiotics from mast cells of hybrid striped bass.

The two elution tubes 16 and 17 showing the greatest bactericidal activity in our studies, isolated by Bio-Gel P-10 fractionation (Fig. 3), did not indicate the presence of bactericidal peptides in granules of channel catfish leucocytes using an SDS–PAGE low molecular weight gel and silver staining for protein (Fig. 2). However, in the data presented here, we did find in catfish leucocytes a low molecular weight non-peptide factor of 655 Da (Fig. 4) that has excellent bactericidal activity against *E. ictaluri*, *A. hydrophila*, and *E. coli* (Table 1), and excellent antiviral activity against herpes simplex virus-type 1. This is an innate antimicrobial factor then that elicits broad-spectrum activity against both bacteria and viruses.

Absorption at 280 nm indicates a conjugated double-bonded aromatic ringed structure. NMR analysis also indicated that the antimicrobial factor was a ringed structure. Only small amounts of the Bio-Gel P-10 purified extract were available, so we were unable to determine its structure by NMR. Elemental analysis found carbon and hydrogen present, but nitrogen was below the level of detection. Elemental analysis effectively rules out a peptide. The solubility of the antimicrobial factor in water indicates the presence of polar groups and that oxygen is likely present, given the absence of nitrogen. By electrospray ionization-MS/MS, the factor ionized much better in the positive mode than in the negative mode, indicating that the factor is a proton acceptor and cationic. No ion fragmentation typical of peptides was seen. This implies then a non-peptide of low molecular weight (655 Da) that may have an isoprenoid-like nucleus or steroid-like nucleus as its primary structure and also contains significant numbers of polar groups with oxygen. Glycosides or polar steroid derivatives are common biologically active molecules that fit the structural profile we have built here.

A water-soluble cationic aminosteroid, called squalamine, has been isolated from various tissues, especially liver and gallbladder, of the dogfish shark [45]. Squalamine has a molecular weight of 628 and exhibits antimicrobial activity against bacteria, fungi, and protozoa [45]. The molecular weight (655 Da) of the cationic non-peptide antimicrobial factor purified here from catfish leucocytes is thus very close in molecular weight to that of squalamine which also is not a peptide. However, squalamine contains more nitrogen than does our compound.

The channel catfish virus (CCV) is the only known pathogenic virus that infects channel catfish [46]. The CCV tends to attack smaller fish like fry and fingerlings rather than larger fish which may be carriers or immune [46]. The virus responsible for CCV has been described as a herpesvirus [47], and the catfish antiviral compound discovered here inactivates a herpesvirus (HSV-1) in tissue culture. The antiviral factor found in channel catfish leucocytes could therefore be important in innate immunity against the CCV.

It is not known if the innate antimicrobial factor isolated here from catfish white blood cells is also secreted and could thereby elicit extracellular killing of *E. ictaluri* in various tissue fluids, mucus, gastrointestinal tract, and plasma. Nutritional factors and environmental stress might also influence production of the antimicrobial factor. If the antimicrobial factor is perhaps innately secreted and in high enough concentration, this might be a reason why blue catfish, certain strains of channel catfish, and channel/blue catfish hybrids are more resistant to *E. ictaluri* infections when compared with less resistant strains of channel catfish [3]. It might prove to be an innate bactericidal factor that could be used in aquaculture for genetic selection in the breeding of channel catfish for resistance to *E. ictaluri* and other infections [3].

Sustainable agriculture should include an understanding of disease resistance and innate immunity of important aquaculture species. More research is there-

fore needed about the defensive responses and innate immunity to infectious diseases by the channel catfish, an important aquaculture species. The novel antimicrobial factor described here from channel catfish leucocytes was both antibacterial and antiviral. A better understanding of resistance mechanisms could lead to better control of fish diseases and thus increased efficiency and production of channel catfish in aquaculture.

The research purified and characterized a low molecular weight non-peptide antimicrobial factor from granules of channel catfish peripheral blood leucocytes. This study of innate immunity should help us better understand how catfish leucocytes kill important pathogenic bacteria and viruses. The information gained might also be helpful in adding to our repertory of antimicrobial compounds that could be useful in treating microbial infections.

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References

[1] J.L. Fryer, J.L. Bartholomew, Established and emerging infectious diseases of fish, ASM News 62 (1996) 592–594.
[2] P.H. Klesius, Immune system of channel catfish: an overture on immunity to *Edwardsiella ictaluri*, in: Annual Review of Fish Diseases, Pergamon Press, New York, NY, 1992, pp. 325–338.
[3] W.R. Wolters, D.J. Wise, P.H. Klesius, Survival and antibody response of channel catfish, blue catfish, and channel catfish female × blue catfish male hybrids after exposure to *Edwardsiella ictaluri*, J. Aquatic Animal Health 8 (1996) 249–254.
[4] A.J. Ainsworth, C. Dexiang, Differences in the phagocytosis of four bacteria by channel catfish neutrophils, Dev. Comp. Immunol. 14 (1990) 201–209.
[5] J.A. Jenkins, D.D. Ourth, Opsonic effect of the alternative complement pathway on channel catfish peripheral blood phagocytes, Vet. Immunol. Immunopathol. 39 (1993) 447–459.
[6] P.R. Waterstrat, A.J. Ainsworth, G. Capley, In vitro response of channel catfish, *Ictalurus punctatus*, neutrophils to *Edwardsiella ictaluri*, Dev. Comp. Immunol. 15 (1991) 53–63.
[7] H.G. Boman, Peptide antibiotics and their role in innate immunity, Annu. Rev. Immunol. 13 (1995) 61–92.
[8] M.J. Manning, Fishes, Immunology: A Comparative Approach, Wiley, New York, NY, 1994, Chapter 3.
[9] H.G. Boman, J. Marsh, J.A. Goode (Eds.), Antimicrobial Peptides, Ciba Foundation Symposium 186, Wiley, New York, NY, 1994.
[10] T. Ganz, R.I. Lehrer, Antimicrobial peptides of leukocytes, Curr. Opin. Hematol. 4 (1997) 53–58.
[11] D.D. Ourth, L.M. Bachinski, Bacterial sialic acid modulates activation of the alternative complement pathway of channel catfish, Dev. Comp. Immunol. 11 (1987) 551–564.
[12] D.D. Ourth, E.A. Wilson, Alternative pathway of complement and bactericidal response of the channel catfish to *Salmonella paratyphi*, Dev. Comp. Immunol. 6 (1982) 75–85.
[13] D.D. Ourth, E.A. Wilson, Agglutination and bactericidal responses of the channel catfish to *Salmonella paratyphi*, Dev. Comp. Immunol. 5 (1981) 261–270.
[14] P.R. Waterstrat, A.J. Ainsworth, G. Capley, Use of a discontinuous Percoll gradient technique for the separation of channel catfish peripheral blood leukocytes, J. Fish Dis. 11 (1988) 289–294.
[15] T. Ganz, M.E. Selsted, D. Szklarek, S.L. Harwig, K. Daher, D.F. Bainton, R.I. Lehrer, Defensins: natural peptide antibiotics of human neutrophils, J. Clin. Invest. 76 (1985) 1427–1435.
[16] T.D. Lockey, D.D. Ourth, Formation of pores in *Escherichia coli* cell membranes by a cecropin isolated from hemolymph of *Heliothis virescens* larvae, Eur. J. Biochem. 236 (1996) 263–271.
[17] D.D. Ourth, T.D. Lockey, H.E. Renis, Induction of cecropin-like and attacin-like antibacterial but not antiviral activity in *Heliothis virescens* larvae, Biochem. Biophys. Res. Commun. 200 (1994) 35–44.
[18] K.T. Chung, D.D. Ourth, Larval and pupal induction and N-terminal amino acid sequence of lysozyme from *Heliothis virescens*, J. Insect Physiol. 46 (2000) 563–572.
[19] T.D. Lockey, D.D. Ourth, Purification and characterization of lysozyme from hemolymph of *Heliothis virescens* larvae, Biochem. Biophys. Res. Commun. 220 (1996) 502–508.
[20] D.D. Ourth, H.E. Renis, Antiviral melanization reaction of *Heliothis virescens* hemolymph against DNA and RNA viruses in vitro, Comp. Biochem. Physiol. 105B (1993) 719–723.
[21] D.O. White, F.J. Fenner, Medical Virology, fourth ed., Academic Press, New York, NY, 1994.
[22] H. Schagger, G. von Jagow, Tricine-sodium dodecyl sulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, Anal. Biochem. 166 (1987) 368–379.
[23] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[24] C. Dass, Principles and Practice of Biological Mass Spectrometry, Wiley, New York, NY, 2001.
[25] D.M. Desiderio (Ed.), Mass Spectrometry of Peptides, CRC Press, Boca Raton, FL, 1991.
[26] J.E. Gabay, Antimicrobial proteins with homology to serine proteases, Antimicrobial Peptides, in: Ciba Foundation Symposium 186, Wiley, New York, NY, 1994, pp. 237–249.
[27] K.T. Chung, D.D. Ourth, Viresin: A novel antibacterial protein from immune hemolymph of *Heliothis virescens* pupae, Eur. J. Biochem. 267 (2000) 677–683.
[28] J.O. Phillips, D.D. Ourth, Isolation and molecular weight determination of two immunoglobulin heavy chains in the channel catfish, Comp. Biochem. Physiol. B 85 (1986) 49–54.
[29] T. Ganz, Biosynthesis of defensins and other antimicrobial peptides, in: Antimicrobial Peptides, Ciba Foundation Symposium 186, Wiley, New York, NY, 1994, pp. 62–76.
[30] D.D. Ourth, Secretory IgM, lysozyme and lymphocytes in the skin mucus of the channel catfish, *Ictalurus punctatus*, Dev. Comp. Immunol. 4 (1980) 65–74.
[31] J.A. Jenkins, D.D. Ourth, Membrane damage to *Escherichia coli* and bactericidal kinetics by the alternative complement pathway

of channel catfish, Comp. Biochem. Physiol. 97B (1990) 477–481.
[32] J.A. Jenkins, R. Rosell, D.D. Ourth, L.B. Coons, Electron microscopy of bactericidal effects produced by the alternative complement pathway of channel catfish, J. Aquatic Animal Health 3 (1991) 16–22.
[33] D.D. Ourth, L.M. Bachinski, Bactericidal response of channel catfish by the classical and alternative complement pathways against bacterial pathogens, J. Appl. Ichthyol. 3 (1987) 42–45.
[34] D.D. Ourth, V.D. Ratts, N.C. Parker, Bactericidal complement activity and concentrations of IgM, transferrin, and protein at different ages of channel catfish, J. Aquatic Animal Health 3 (1991) 274–280.
[35] D.D. Ourth, E.A. Wilson, Bactericidal serum response of the channel catfish against gram-negative bacteria, Dev. Comp. Immunol. 6 (1982) 579–583.
[36] R.A. Goldsby, T.J. Kindt, B.A. Osborne, J. Kuby, Immunology, fifth ed., W.H. Freeman, New York, NY, 2003, Chapter 2.
[37] J.E. Gabay, R.W. Scott, D. Campanelli, Antibiotic proteins of human polymorphonuclear leukocytes, Proc. Natl. Acad. Sci. USA 86 (1989) 5610–5614.
[38] E. Martin, T. Ganz, R.I. Lehrer, Defensins and other endogenous peptide antibiotics of vertebrates, J. Leukoc. Biol. 58 (1995) 128–136.
[39] A.M. Cole, P. Weisand, G. Diamond, Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder, J. Biol. Chem. 272 (1997) 12008–12013.
[40] S.E. Douglas, J.W. Gallant, Z. Gong, C. Hew, Cloning and developmental expression of a family of pleurocidin-like antimicrobial peptides from winter flounder, *Pleuronectes americanus*, Dev. Comp. Immunol. 25 (2001) 137–147.
[41] D.W. Robinette, S. Wade, T. Arroll, M. Levy, W. Miller, E.J. Noga, Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like antimicrobial proteins, Cell Mol. Life Sci. 54 (1998) 467–475.
[42] R.C. Richards, D.B. O'Neil, P. Thibault, K.V. Ewart, Histone H1: an antimicrobial protein of Atlantic salmon (*Salmo salar*), Biochem. Biophys. Res. Commun. 284 (2001) 549–555.
[43] J.H. Cho, I.Y. Park, H.S. Kim, W.T. Lee, M.S. Kim, S.C. Kim, Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish, FASEB J. 16 (2002) 429–431.
[44] U. Silphaduang, E.J. Noga, Peptide antibiotics in mast cells of fish, Nature 414 (2001) 268–269.
[45] K.S. Moore, S. Wehrli, H. Roder, M. Rogers, J.N. Forrest, D. McCrimmon, M. Zasloff, Squalamine: an aminosterol antibiotic from the shark, Proc. Natl. Acad. Sci. USA 90 (1993) 1354–1358.
[46] R.R. Stickney, Principles of Aquaculture, Wiley, New York, NY, 1994, pp. 427–429.
[47] K. Wolf, R.W. Darlington, Channel catfish virus: a new herpesvirus of ictalurid fishes, J. Virol. 8 (1971) 525–533.