

Purification of a novel arthropod defensin from the American oyster, *Crassostrea virginica*

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Abstract

An antimicrobial peptide was purified from acidified gill extract of a bivalve mollusk, the American oyster (*Crassostrea virginica*), by preparative acid-urea–polyacrylamide gel electrophoresis and reversed-phase high performance liquid chromatography. The 4265.0 Da peptide had 38 amino acids, including 6 cysteines. It showed strongest activity against Gram-positive bacteria (*Lactococcus lactis* subsp. *lactis* and *Staphylococcus aureus*; minimum effective concentrations [MECs] 2.4 and 3.0 µg/ml, respectively) but also had significant activity against Gram-negative bacteria (*Escherichia coli* D31 and *Vibrio parahaemolyticus*; MECs 7.6 and 15.0 µg/ml, respectively). Comparison of the amino acid sequence with those of other known antimicrobial peptides revealed that the novel peptide had high sequence homology to arthropod defensins, including those from other bivalves, the mussels *Mytilus edulis* and *Mytilus galloprovincialis*. This is the first antimicrobial peptide to be isolated from any oyster species and we have named it American oyster defensin (AOD). © 2005 Elsevier Inc. All rights reserved.

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Marine invertebrates have no specific, adaptive immunity, but only innate defenses, including cellular immunity (e.g., phagocytosis, encapsulation, and respiratory burst) mediated by immune cells (e.g., hemocytes) and humoral immunity (e.g., lectins, agglutinins, and lysosomal enzymes) in host compartments such as plasma [1,2]. There is increasing evidence that antimicrobial peptides (AMP) are a common component of the immune defenses in marine invertebrates and a number of AMP have recently been reported from marine invertebrates including chelicerates (horseshoe crabs [3,4]), crustaceans (penaeid shrimp [5], blue crab [6], shore crab [7], and woodlouse [8]), and urochordates (solitary tunicates [9,10]). In mollusks, AMP have been described from a gastropod, the sea hare *Dolabella auricularia* [11], as well as two bivalves, the mussels *Mytilus edulis* [12] and *Mytilus galloprovincialis* [13,14].

The American oyster (*Crassostrea virginica*), a bivalve mollusk, is a keystone species that is very economically important and plays a critical role in estuarine ecosystems by virtue of its tremendous capacity to filter water, removing algae and other suspended matter. It also provides habitat for an enormous range of other animals (e.g., worms, snails, sea squirts, sponges, crabs, fish, etc.) and provides a food source for many of them. Filter-feeding bivalves like oysters live in microbe-rich environments and are constantly exposed to high concentration of often pathogenic microbes [15]. To combat these potential invaders, innate defenses such as constitutively expressed AMP might play a critical role. However, to date, no antimicrobial peptide has been isolated from any oyster species [16], even though, as a group, oysters are the most economically important mollusks worldwide. While a number of pathogens, especially bacteria and parasites, have devastated American oyster populations, relatively little is known about their innate immune defenses. Here we report for the first time

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the isolation and initial characterization of an AMP from the American oyster.

Materials and methods

Tissue extraction. Twenty-three adult oysters, 7.0–12.0 cm in shell length, were obtained from a local seafood market in Raleigh, North Carolina, USA. The oysters, collected in November 2004, originated from the Gulf of Mexico near Louisiana, USA. As soon as they arrived in the laboratory, each oyster was processed individually. The shell was gently washed with tap water and was then opened with a shucking knife. Gill was then harvested and placed into a 15 ml sterile polypropylene tube on wet ice until 5 ml of gill tissue from a total of 4–5 oysters was harvested. The gill tissue was then added to a 50 ml beaker having 15 ml of deionized water containing protease inhibitor cocktail (PIC, Sigma # P-8340, St. Louis, MO, USA) on wet ice to inhibit protein degradation. The PIC was at four times the standard concentration recommended for mammalian tissues (final concentrations were 4.95 mM AEBSF, 3.8 μ M aprotinin, 95.2 μ M leupeptin, 190 μ M bestatin, 71.4 μ M pepstatin A, and 66.6 μ M E-64). Immediately after adding the gill tissue, 150 μ l of glacial acetic acid (HAc) (final concentration of 1% HAc) was added. The tissue was immediately homogenized on wet ice for 5 min (speed #3, Polytron PT1200 C homogenizer, Kinematica AG, Switzerland). After homogenization, the extract was boiled for 5 min and then stored on wet ice. A total of five boiled samples (23 oysters total) were centrifuged at 15,000g for 45 min at 4 °C. The supernatants were pooled (total of 79 ml) and stored at –70 °C until use.

Proteolytic digestion of crude extract to determine if antimicrobial compounds were proteinaceous. Susceptibility of the antibacterial activity of crude gill extract to proteolytic digestion was determined by incubation of gill extract with 250 μ g/ml crystalline trypsin (Fisher Scientific, Fair lawn, NJ) for 60 min at 37 °C. Extract was prepared as described above except PIC was omitted. Antibacterial testing of the extract before and after protease treatment was done with the standard radial diffusion assay against *Escherichia coli* D31.

Standard radial diffusion assay. Antibacterial activity during purification was assessed with the radial diffusion assay as described by Hultmark et al. [17]. Briefly, an overnight culture (18 h) of *E. coli* D31 grown at 37 °C in trypticase soy broth with 1% NaCl was washed three times by centrifuging at 590g for 10 min at 4 °C followed by suspension with cold, phosphate-buffered saline (pH 7.0). The bacterial suspension was adjusted to an optical density (OD₅₇₀) of 0.1 (10⁸ CFU/ml). One milliliter of the bacterial suspension was added to autoclaved Luria–Bertani broth containing 1.5% low EEO agarose, 0.5% NaCl, 200 mM phosphate buffer (pH 6.7), and 100 μ g/ml streptomycin sulfate, which was incubated in a water bath at 48 °C. Ten milliliters of the bacterial suspension was poured into sterile 100 cm² Petri dishes with a 10 mm grid (Falcon 1112). For radial diffusion assays, 3 μ l of sample was pipetted into 2.5 mm diameter wells and the plate was incubated at 37 °C for 18 h, at which time clearing zone diameters were measured to the nearest 0.1 mm. When assessing the amount of activity during various purification steps, units of antibacterial activity were calculated as described previously [18] from the clearing zone diameters of test samples by using a standard curve of a pure polypeptide antibiotic (calf histone H2B).

Ultrasensitive radial diffusion assay. The antibacterial activity of the purified peptide was assessed with a modified version of the double-layer radial diffusion assay as described by Lehrer et al. [19], since this assay generally requires the least amount of material. We tested both crude extract and purified peptide against *E. coli* D31, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, and *Lactococcus lactis* subsp. *lactis*. All were grown overnight for 18 h in trypticase soy broth (TSB) at the appropriate temperature (37 °C for *E. coli* D31, *L. lactis* subsp. *lactis*, *S. aureus*, and 25 °C for *V. parahaemolyticus*); 1% NaCl was also added to the medium for *V. parahaemolyticus*. After overnight incubation, the bacterial suspension was diluted to a McFarland turbidity standard of 0.5 (Vitek Colorimeter #52-1210, Hach, Loveland, Colorado) corresponding to \sim 10⁸ CFU/ml. One-half milliliter of diluted bacterial suspension was added to 9.5 ml of

underlay gel containing 5 \times 10⁶ CFU/ml in 10 mM phosphate buffer (pH 6.57) with 0.03% TSB and 1% Type I (low EEO) agarose; 1% NaCl was also added to the medium for *V. parahaemolyticus*. The purified peptide was serially diluted 2-fold in acidified water (0.01% HAc) and each dilution was added to 2.5 mm diameter wells made in the 1 mm thick underlay gels. After incubation for 3 hr at either 25 °C (for *V. parahaemolyticus*) or 37 °C (*E. coli* D31, *S. aureus*, *L. lactis* subsp. *lactis*), the bacterial suspension was overlaid with 10 ml of double-strength overlay gel containing 6% TSB with 10 mM phosphate buffer (pH 6.57) in 1% agarose. Plates were incubated for an additional 18–24 h and then the clearing zone diameters were measured. After subtracting the diameter of the well, the clearing zone diameter was expressed in units (0.1 mm = 1 U). The MEC (minimal effective concentration, μ g/ml) of the purified peptide was calculated as the X-intercept of a plot of units against the log₁₀ of the peptide concentration [20]. Piscidin 1, an α -helical AMP isolated from hybrid striped bass (*Morone saxatilis* \times *M. chrysops*) [21], was used as a positive control.

Acid-urea PAGE (AU–PAGE). Acid-urea–polyacrylamide gel electrophoresis was performed to monitor purity of the AMP at various steps. Gels (100 mm wide \times 75 mm long \times 1.0 mm thick) were prepared having 12% acrylamide; 37.5/1 (w/w) acrylamide/bis solution, 4.8 M urea, 5% acetic acid (HAc), 0.48% (v/v) *N,N,N',N'*-tetramethylethylenediamine (TEMED), and 0.22% (w/v) ammonium persulfate (APS). The gel was polymerized at room temperature overnight. The APS and TEMED were removed by pre-running the gel with 5% acetic acid for 60 min at 150 V with reversed polarity (lower-chamber cathode) using a Mini PROTEAN II Electrophoresis Cell (Bio-Rad Laboratories, Richmond, CA). No stacking gel was used. Samples were mixed (1:1) with sample solution (3.0 M urea in 5% HAc containing methyl green as a tracking dye) and electrophoresed with 5% HAc at 150 V for 50 min at room temperature. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 dye and appropriately destained in MeOH:HAc:H₂O (4:1:5).

Continuous AU–PAGE. Crude gill extract containing PIC was initially purified by preparative continuous acid-urea–polyacrylamide gel electrophoresis (CAU–PAGE) using a Bio-Rad Prep Cell 491 (Bio-Rad, Laboratories, Richmond, CA) as described by Harwig et al. [22]. A 6.8 cm separating gel (12% acrylamide; 37.5/1 w/w acrylamide/bis solution) containing 5% HAc and 4.8 M urea was polymerized overnight in the 28 mm diameter tube of the Prep Cell apparatus. The catalyst concentrations were 0.48% (v/v) TEMED and 0.22% (w/v) APS. No stacking gel was used. Gels were pre-run for 90 min at 4 °C at a constant amperage (40 mA) with reversed polarity (lower-chamber cathode). Both the upper and the lower chambers contained 5% HAc. Following pre-running, the upper chamber was refilled with 5% HAc. Ten milliliters of acidified gill extract was mixed with 5 ml of sample solution (3.0 M urea in 5% HAc), layered onto the gel, and electrophoresed at a constant current of 30 mA in 5% HAc at 4 °C. The sample was eluted at a flow rate of 0.8 ml/min and 12 ml fractions were collected for 12.5 h (600 ml of eluate). The fractions were stored at –70 °C overnight and then lyophilized. After lyophilization, each fraction was dissolved in 100 μ l of 0.01% HAc and 3 μ l of each fraction was tested for antibacterial activity against *E. coli* D31 with the standard radial diffusion assay.

Reversed-phase HPLC. Fractions showing antibacterial activity with CAU–PAGE were pooled and loaded onto a C₄ reversed-phase column (Jupiter 5 μ m, 300 Å, 4.6 \times 250 mm, Phenomenex, Torrance, CA, USA). The sample was eluted with an isocratic gradient of 7% acetonitrile in 0.1% TFA for 10 min followed by a linear gradient of 7–70% acetonitrile in 0.1% TFA for 45 min at a flow rate of 1 ml/min. The eluate was monitored at 214 nm using a Waters 486 UV detector. Fractions were hand collected, dried under vacuum, dissolved in 0.1% TFA, and tested for antibacterial activity against *E. coli* D31 by the standard radial diffusion assay. The fraction containing the greatest activity was rechromatographed under the same conditions.

Structure determination. Electrospray ionization mass spectrometry (ESI–MS) data were obtained using a quadrupole time-of-flight (Q–TOF) mass spectrometer (Waters/Micromass, Manchester, UK). Prior to analysis, a small aliquot of the purified peptide was diluted with 1% formic acid/50% acetonitrile/water to approximately 5 pmol/ μ l. This was injected into the mass spectrometer using a nanospray ionization source equipped

with a PicoTip Emitter (#BG10-78-4-CE20, New Objectives, Woburn, MA). The mass spectral data were obtained in the positive mode and transformed to the average molecular mass using the MaxEnt algorithm. Automated Edman chemical degradation of the intact purified peptide was performed according to the manufacturer's protocol on a Procise 494 cLc Peptide/Protein Sequencer (Applied Biosystems, Foster City, CA) equipped with an on-line HPLC system.

To reduce and alkylate the purified peptide, 50 μ l of 8 M urea with 0.1 M ammonium bicarbonate and 5 μ l of 45 mM dithiothreitol were added to 500 pmol of the (dried) purified peptide. The mixture was incubated for 30 min at 37 °C. After the sample had cooled to room temperature, 5 μ l of 100 mM iodoacetamide was added and incubated for 30 min at room temperature. An aliquot was taken to determine the carbamidomethylated (monoisotopic) mass of the intact protein by ESI-MS on an APEX-Q 9.4 Tesla Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer (Bruker Daltonics, Billerica, MA).

In order to concentrate the carbamidomethylated sample prior to tryptic digestion, and to ensure 100% cysteine modification, 20 μ l (50 pmol) was bound to a C18 ZipTip (Millipore, Billerica, MA), eluted in 10 μ l of 0.1% TFA/50% acetonitrile/water, and dried. The aliquot was then dissolved in 2 μ l of 8 M urea/0.1 M ammonium bicarbonate. Two microliters of TCEP was added and the aliquot was incubated for 30 min at 37 °C. After the sample had cooled to room temperature, 2 μ l of 100 mM iodoacetamide was added and incubated for 30 min at room temperature. Three microliters of water was added along with 1 μ l of 0.1 mg/ml sequencing grade modified trypsin (Promega, Madison, WI) and then incubated overnight at 37 °C. Enzymatic cleavage was confirmed by running 1 μ l of the digest mixture on a Matrix Assisted Laser Desorption Ionization mass spectrometer (MALDI L/R, Waters/Micromass, Manchester, UK) after ZipTip cleanup as above. The intact protein mass observed on the FT-ICR was not evident, indicating the peptide had been digested. Another 1 μ l of the digest was injected onto a 100 μ m \times 150 mm Atlantic dC18 NanoEase column (Waters) for LC-MS/MS sequencing on a Q-ToF API mass spectrometer (Waters/Micromass, Manchester, UK). A second 1 μ l aliquot of the digest was also desalted using a C18 ZipTip as described above and analyzed by MALDI-ToF-ToF (Applied Biosystems, model 4700) for both MS and MS/MS acquisition. The remaining digest was separated by RP-HPLC on a Vydac C18 1 mm \times 250 mm column eluted at 50 μ l/min. UV-absorbing peaks were further analyzed by MALDI-MS prior to potential additional Edman chemical sequencing.

G F G X P W N R Y Q X H S H X R S I G R L G G Y X A G S L R L T X X X Y R
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37

Sequence analysis. Homology search of the purified peptide was performed using BLASTP 2.2.10 and TBLASTN 2.2.10 on Genome Net (<http://www.ncbi.nlm.gov/BLAST>). The theoretical isoelectric point (pI) and molecular mass were estimated by ExPASy (<http://www.expasy.ch/tools/peptide-mass.html>). Sequence alignment was performed with the ClustalX program [23].

Results

Peptide purification

Initial screening of acidified, crude extract (without protease inhibitors) from American oyster gill showed strong activity against both Gram-positive (*S. aureus* and *L. lactis* sub. *lactis*) and Gram-negative (*E. coli* D31 and *V. parahaemolyticus*) bacteria using the ultrasensitive radial diffusion assay. The MEC of all four isolates was 0.06–0.13 μ l. Since the gill tissue was diluted 1:4 with 1% HAc, the MEC of

undiluted tissue was probably ~0.015–0.033 μ l. The acetic acid had no antibacterial activity. Treatment of extract with trypsin for 90 min at 37 °C completely abolished all antibacterial activity against *E. coli* D31 (data not shown; other bacteria were not tested), suggesting that the extract contained a proteinaceous antibiotic. Thus, to inhibit proteolysis, protease inhibitor cocktail was added during subsequent preparations of extract for antibiotic isolation.

The antibacterial activity was purified with CAU-PAGE followed by RP-HPLC. The activity after CAU-PAGE separated into two distinct zones, a rapid (fractions 15–16) and a slow (fractions 23–28) migrating zone (Fig. 1). The active fractions of the rapid migrating zone from two CAU-PAGE runs (10 ml crude extract per run) were pooled and subjected to C₄ RP-HPLC. Antibacterial activity was present in several peaks; the peak with the strongest activity eluted at 41% acetonitrile (Fig. 2). Recycling this fraction showed a single peak on RP-HPLC (data not shown). Acid-urea-PAGE of the purified peptide showed a single band that migrated slightly slower than piscidin 1 (Fig. 3A). Purification of 20 ml of oyster extract yielded 35.9 μ g of the purified peptide. We used 14.4 μ g of the purified peptide for chemical characterization. Several other peaks also displayed antibacterial activity and they are currently being characterized.

Peptide identification

Electrospray ionization mass spectroscopy (ESI-MS) of the purified, unreduced peptide yielded an average isotopic mass of 4265.0 Da (Fig. 3B). Edman degradation suggested that the peptide consisted of 37 residues, with no phenylthiohydantoin (PTH) amino acids observed at positions 4, 11, 15, 25, 33, 34, and 35:

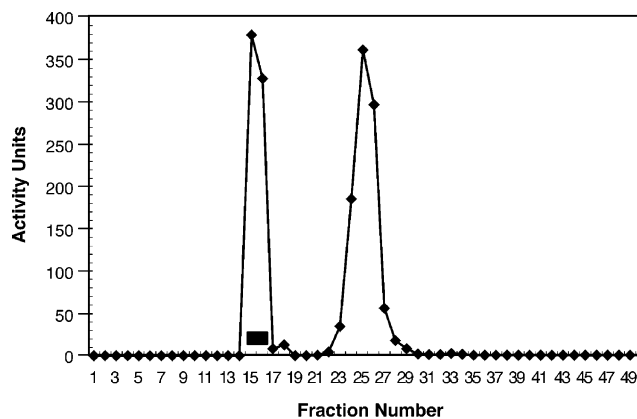


Fig. 1. Recovery of antibacterial activity during CAU-PAGE. Ten milliliters of crude gill extract was loaded onto the column. Each fraction represents 12 ml of eluate. For isolation of American oyster defensin, fractions 15–16 were pooled (black bar).

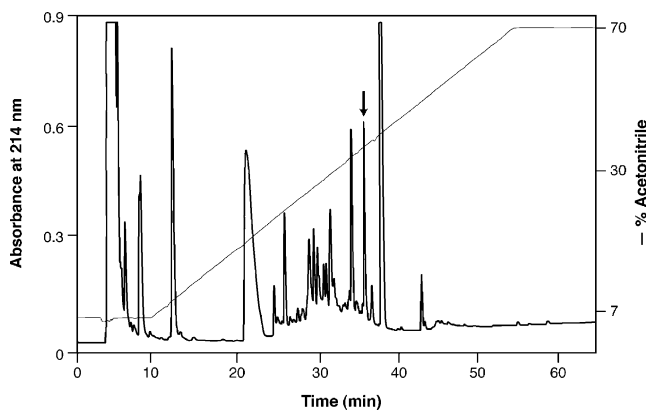


Fig. 2. Reversed-phase HPLC separation of 200 μ l of semi-purified oyster gill extract. Acidic extract was fractionated by continuous AU-PAGE. Active fractions were pooled and then applied to a C_4 reversed-phase column. The elution point of American oyster defensin was at 41% ACN (arrow).

An unmodified cysteine would not be observed during Edman sequencing and thus, the lack of an observed PTH amino acid suggested that the peptide contained several cysteines. To determine the number of cysteine residues, carbamidomethylated peptide was run on the FT-ICR MS, yielding a monoisotopic mass of 4610.04 Da, which differed by 345 Da from the average mass (4265.0 Da) via Q-ToF ESI-MS of the non-carbamidomethylated peptide. Since carbamidomethylation would add 57 Da to the mass of each cysteine residue, this mass difference suggested the presence of six cysteines. To confirm the number and positions of cysteines and the other unconfirmed residues, the native peptide was digested with trypsin after reduction and alkylation, followed by MALDI-MS screening to confirm cleavage and then LC-MS/MS and MALDI-Tof-Tof of the digested fragments. In parallel, another aliquot of the digested fragments was separated by RP-HPLC and the peaks were screened by MALDI-MS.

Analysis of the LC-MS/MS spectra, combined with an automated Mascot search, revealed the sequence of three tryptic peptide fragments (residues 1–8, 21–30, and 31–37). One peptide fragment (residues 31–37) was matched to a defensin and we identified the amino acid at position 34 as Thr and at positions 33 and 35 as cysteine. Cysteine residues were also verified at positions 4 and 25. A doubly charged mass of 574.23 Da, which corresponded to the predicted tryptic mass for residues 9–16 with two carbamidomethylated cysteines, was detected, but did not fragment. The data obtained on the MALDI-Tof-Tof analysis of the digest also contained these three peptide sequences, along with sequence verification of residues 12–16, with residue 15 containing a cysteine. By combining the Edman sequencing results, which indicated that the tryptic peptide spanning residues 9–16 began with YQ (positions 9 and 10), with the MALDI-Tof-Tof MS and MS/MS data, the only possible amino acid for residue 11 is a cysteine.

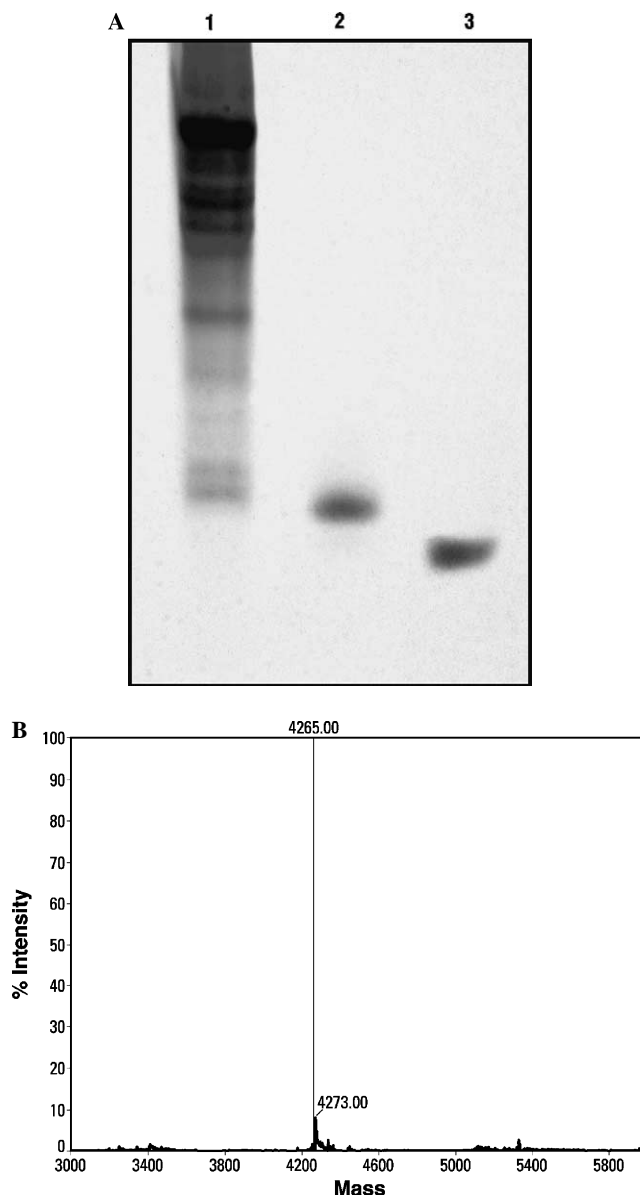


Fig. 3. (A) Acid-urea-PAGE of crude gill extract and purified American oyster defensin (AOD) stained with Coomassie blue. Lane 1, crude gill extract (15 μ l); lane 2, AOD (1 μ g, M.W. 4265); lane 3, another AMP (piscidin 1), with the same amount loaded as AOD (1 μ g, M.W. 2543). (B) Electrospray ionization mass spectroscopy (ESI-MS) of the purified American oyster defensin. The transformed spectrum shows a mass of 4265.0 Da.

Two peptides of interest were located by linear MALDI-MS screening of the HPLC peaks. The first [with mass of 1148.2 Da average protonated mass = 1034.17 predicted protonated mass + (57 \times 2)] corresponded to the molecular weight of residues 9–16 containing two carbamidomethylated cysteines. The second [974.19 Da average protonated mass = 860.05 + (57 \times 2)] corresponded to the molecular weight of residues 31–37. The monoisotopic mass of the intact carbamidomethylated sequence, in which six unidentified residues were Cys and one unidentified residue was Thr, as determined by MS and Edman degradation was

4523.01 Da. There was an 87 Da difference between this mass and the mass determined by FT-ICR MS (4610.0 Da). The difference of 87 Da suggested that there was a C-terminal serine (87.08 Da) as a 38th residue. The combined data lead us to conclude that the peptide was without secondary modification and had the following sequence:

G F G C P W N R Y Q C H S H C R S I G R L G G Y C A G S L R L T C T C Y R S
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38

The calculated average mass of this sequence with the cysteines in disulfide bonds (4264.88 Da) was in excellent agreement with the average isotopic mass (4265.0 Da) originally obtained by ESI-MS. The sequence consists of 38 residues including six cysteines and seven basic residues (including His and Arg). The calculated isoelectric point (pI) of the purified peptide was 9.18. Comparison of the amino acid sequence with those of other known antimicrobial peptides using BLASTP 2.2.10 and TBLASTN 2.2.10 of Genome Net did not reveal identity with any known or deduced polypeptide sequence. However, the sequence showed a high degree of similarity with arthropod defensins and was thus named American oyster defensin (AOD) (Fig. 4).

Primary structure relationships

The AOD sequence was aligned with eight closely related published sequences (Fig. 4). Allowing for several gaps, it was apparent that the cysteine array was comparable in all these peptides, although MGD-1 and MGD-2 possess two additional cysteines. Sequence homology search with BLASTP 2.2.10 showed that American oyster defensin was highly similar to the sequences of defensins [24] from primitive arthropods including those from a dragonfly [*Aeschna cyanea* (68%)] [25], two scorpions [*Leiurus quinquestriatus* (68%)] [26], *Androctonus australis* (70%) [27], and a soft tick [*Ornithodoros moubata*] (62%) [28]. The American oyster defensin also showed high sequence homology with defensins from the mussels, *M. edulis* (defensin A, 71% and defensin B, 69%) and *M. galloprovincialis* (MGD-1, 73% and MGD-2, 71%) [12–14]. All these defensins are highly basic with calculated isoelectric points ranging from 8.92 to 9.69.

Antibacterial activity

The antibacterial activity of AOD with the ultrasensitive radial diffusion assay was somewhat stronger than that of piscidin 1 against the Gram-positive bacteria, *L. lactis* subsp. *lactis* and *S. aureus*. Significant activity, similar to that of piscidin 1, was also observed against the two Gram-negative

bacteria, *E. coli* D31 and *V. parahemolyticus* (Table 1).

Discussion

Although both cultured and feral American oyster populations are susceptible to many infectious agents including bacteria such as vibrios [29], antimicrobial peptides have not been previously reported from this species. Here, we report a novel member of the arthropod defensin family that is apparently constitutively expressed, since it was present in presumably healthy oysters without experimental microbial challenge. Peptide purification was accomplished by preparative continuous AU-PAGE and reversed-phase HPLC using a C₄ column (Figs. 1 and 2). The purified peptide, named American oyster defensin (AOD), is a 4265.0 Da peptide (Fig. 3) having 38 amino acids with six cysteines that presumably form three intramolecular disulfide bridges as has been shown for some other arthropod defensins.

As shown in Fig. 4, AOD had high sequence homology (62–73%) with various arthropod defensins, including those from dragonfly *A. cyanea* [25], scorpions *L. quinquestriatus* [26] and *A. australis* [27], soft tick *O. moubata* [28], and mussels *M. edulis*, *M. galloprovincialis* [12–14]. All (including AOD) typically share six invariant cysteine residues, several basic amino acid residues (at least +4, basic residues including His and Arg), an amino terminal hydrophobic loop region ‘Gly-Phe-Gly-Cys-Pro,’ a tetra amino acid motif ‘Gly-Gly-Tyr-Cys,’ and a carboxy terminal penta amino acid motif ‘Cys-Thr-Cys-Tyr-Arg.’ They also contain several Gly residues and one or two Pro residues, mainly located in loop and turn regions. The three-dimensional solution structure of *M. galloprovincialis* defensin 1 (MGD-1) by ¹H NMR showed that it consists of an

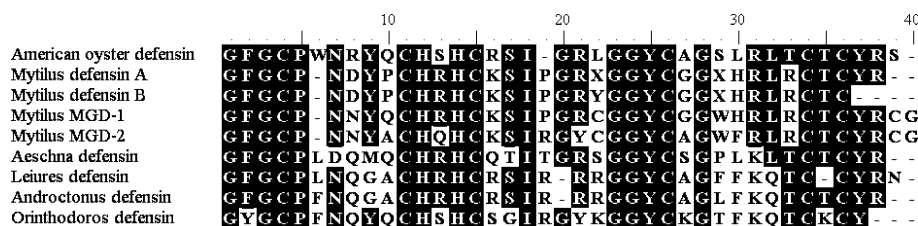


Fig. 4. Alignment of the American oyster defensin amino acid sequence with other arthropod defensin sequences: mussel defensins A and B from *M. edulis* [12], MGD-1 and MGD-2 from *M. galloprovincialis* [13,14], dragonfly defensin from *A. cyanea* [25], scorpion defensins from *L. quinquestriatus* [26] and *A. australis* [27], and soft tick defensin from *O. moubata* [28]. Conserved amino acids are indicated by black boxes.

Table 1
Minimal effective concentration (MEC) of American oyster defensin and piscidin 1 against Gram-positive and Gram-negative bacteria

Bacterium	Gram	MEC ($\mu\text{g/ml}$) [μM]	
		American oyster defensin	Piscidin 1
<i>L. lactis</i> subsp. <i>lactis</i>	+	3.0 [0.7]	12.0 [4.7]
<i>S. aureus</i>	+	2.4 [0.6]	12.5 [4.9]
<i>E. coli</i> D31	–	7.6 [1.8]	4.4 [1.7]
<i>V. parahemolyticus</i>	–	15.0 [3.5]	13.0 [5.1]

α -helical segment (Asn⁷-Ser¹⁶) and two antiparallel β -strands (Arg²⁰-Cys²⁵ and Cys³³-Arg³⁷), together giving rise to the common cystine-stabilized α - β motif (CS $\alpha\beta$ motif) observed in scorpion toxins [30]. The solution structure of MGD-1 infers that AOD might have the CS $\alpha\beta$ motif consisting of an α -helix (Arg⁸-Ser¹⁷) and two antiparallel β -strands (Arg²⁰-Cys²⁵ and Cys³³-Arg³⁷). The primary structure of AOD had a very conserved consensus sequence pattern seen with most arthropod defensins [13,26]:

Cys¹-[---]-Cys²-Xaa-Xaa-Xaa-Cys³-[---]-Gly-Xaa-Cys⁴-[---]-Cys⁵-Xaa-Cys⁶

These common structural features indicate that AOD is a member of the arthropod defensin family.

While AOD showed strongest antibacterial activity against Gram-positive bacteria, it also had surprisingly significant activity against Gram-negative bacteria, including both *E. coli* D31 and *V. parahemolyticus*, suggesting that AOD might have a quite broad antibacterial spectrum compared to those of most other arthropod defensins which typically have strong antibacterial activity against a wide range of Gram-positive bacteria but very weak or no activity against Gram-negative bacteria [24–26,28]. However, native MGD-1, which had high sequence homology with AOD, also showed significant activity against Gram-negative bacteria including *E. coli*, *Vibrio alginolyticus*, *Vibrio splendidus*, and *Vibrio* P1 with minimum inhibitory concentrations (MIC) of 1.6–6.4 $\mu\text{g/ml}$ [13]. In contrast, while synthetic MGD-1 showed similarly strong activity against Gram-positive bacteria including *S. aureus* and *Micrococcus luteus* (MIC of 2.6 $\mu\text{g/ml}$ for both), it appeared to have weaker activity against certain Gram-negative bacteria (i.e., *V. alginolyticus*, with MIC \sim 220 $\mu\text{g/ml}$) [30]. The reason for this difference in activity between native and synthetic MGD-1 was not explained. While native MGD-1 has a hydroxylated tryptophan at position 28 and synthetic MGD-1 is unmodified, this does not appear to affect the activity of synthetic MGD-1 against the other Gram-positive or Gram-negative bacteria that were tested. Furthermore, AOD is not modified.

The American oyster defensin is highly basic, with a calculated pI of 9.18, and has a large hydrophobic region comprising the Phe², Pro⁵, Trp⁶, Tyr⁹, Ile¹⁸, Ala²⁶, Leu²⁹, and Leu³¹ residues that are most likely configured into helix and loop regions. The distribution of hydrophobic and charged residues has a crucial influence on the antimicrobial activity of AMP [30]. Studies have

shown that the soft tick defensin permeabilizes the cytoplasmic membrane, causing lysis of Gram-positive bacteria [31]. While further studies are needed, the potent and relatively broad antibacterial activity of AOD suggests that its configuration presumably facilitates interaction with both Gram-positive and Gram-negative bacterial membranes.

In previous studies, when Jenny et al. [32] screened the ESTs from a hemocyte library of American oyster using a conserved defensin motif, a clone that appears to code for an AMP was identified. This clone (GenBank Accession No. BG624524) appeared to code for part of a sequence that had homology to big defensin, an AMP unrelated to arthropod defensins that had previously been isolated from the hemocyte granules of the Japanese horse-shoe crab, *Tachypleus tridentatus* [32].

A commonality between vertebrate and invertebrate AMPs is that, when they originate from circulating blood cells, they are usually constitutively expressed and stored in these cells [16]. Since we purified AOD from unchallenged oysters that contain many hemocytes in the gill filaments, it is possible that AOD might be constitutively expressed in hemocytes, as are mussel defensins [14].

In conclusion, we isolated an antimicrobial peptide from American oyster, which we have named American oyster defensin (AOD), that has strong activity against both Gram-positive and Gram-negative bacteria. This peptide might be helpful in selecting disease resistant oysters for aquaculture and fisheries. Also, our results might be used to better understand the innate immune system of American oysters and to enhance research to protect it from important microbial infections. Further proteomic and genomic studies are needed to identify sites of synthesis and storage of AOD and determine mechanisms affecting its regulation.

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