Curr Microbiol (2012) 64:501–505
DOI 10.1007/s00284-012-0096-5

Screening of Antimicrobials from Caribbean Sea Animals and Isolation of Bactericidal Proteins from the Littoral Mollusk *Cenchritis muricatus*

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Received: 29 November 2011 / Accepted: 3 February 2012 / Published online: 26 February 2012

Abstract Marine organisms represent approximately half of the world's biodiversity by virtue of the sea being an immense reservoir of bioactive molecules. Here, antimicrobial crude extract activities of different marine invertebrates from the Caribbean Sea were evaluated. One of the most active, crude extracts was that marine snail *Cenchritis muricatus*, it was capable of totally inhibiting the development of *Staphylococcus aureus* and also showed a growth inhibition of 95.9% in *Escherichia coli*. Aiming to isolate molecules that confirm antimicrobial activity, the crude extract was purified by reversed-phase HPLC C-18 chromatography. Thereafter, one of the obtained fractions preserved this antibacterial activity. Furthermore, SDS-PAGE analysis (15%) showed the presence of two proteins of molecular masses with approximately 10 and 15 kDa, respectively. The first 19 amino acids of both proteins were sequenced by using Edman degradation, yielding unidentified primary structures compared against sequences deposited at NCBI databank. This is the first report of antibacterial proteins isolated from the mollusk *Cenchritis muricatus* and these proteins could be used as antibiotic alternatives in the aquacultural industry, as well as in agricultural or biomedical research.

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Introduction

Nowadays, antibiotic resistance developed by bacterial pathogens is one of the major health problems that affect all countries [3]. Therefore, the development of newer antimicrobial therapies that diminishes this resistance is urgent [8]. Antimicrobial peptides (AMPs), a controllable set of peptides evolutionary conserved to fight infections in all forms of life, could be a reassuring solution to this problem [27]. Many AMPs could combine antimicrobial activity with immunomodulatory and anti-inflammatory activities [24]. Although bacteria have resistance mechanisms against these peptides, the multifunctionality of AMPs could evade bacterial resistance [26]. In fact, as hosts and pathogens have co-evolved since ancient times, AMPs have constituted an effective component of the innate immune system [22].

AMPs exhibit a broad spectrum of activity against a wide range of microorganisms [15, 20]. Different mechanisms of action have been proposed for these molecules [4, 13], which indicate that many of them could have more than one antimicrobial target at the cellular level [7]. There are multiple sources of antimicrobial peptides, including plants, mammals, and invertebrates [27]. In spite of this, recent advances in the discovery of antimicrobial peptides in marine invertebrates have shown few described cases [10, 21]. The requirement of scuba diving training for the collection organisms has precluded any major development

in the research of natural marine products [19]. Otherwise, the search for novel antimicrobials in marine invertebrates is extremely interesting and desirable, since invertebrates do not have an adaptive system. In fact they essentially use their humoral defense on a wide and effective group of AMPs that may directly interact with microbes or their toxic molecules [21]. Owing to this fact, many researchers had used marine invertebrates as sources for the development of novel antimicrobials [10, 21].

In this study, a screening of antibacterial activity of marine invertebrates from the Caribbean Sea was performed. Aiming to contribute toward this field of research, antibacterial proteins from the littoral snail *Cenchritis muricatus* were here isolated and identified.

Materials and Methods

Biological Materials

Budonosoma granulifera, *Cassiopeia xamachana*, *Chloeia fusca*, *Condylactis gigantea*, *Echinaster echinophorus*, *Eunicea calyculata*, *Holothuria mexicana*, *Lissodendoryx isodictyalis*, *Ophiocoma echinata*, *Palythoa caribaeorum*, *Phallusia nigra*, *Plexaurella dichotoma*, *Xetosporangia muta*, and *Zoobotryum vesiculariidae* were collected on the northern coast of Cuba near Havana. Collections were made at depths of 3–5 m. Specifically, *Cenchritis muricatus* specimen were hand-collected from the seacoast rocks above the high tide line of Jibacoa beach. *Escherichia coli* (ATCC 8739) strain was obtained from the collection American Type Culture and *Staphylococcus aureus* was clinically isolated and kindly donated by the University of Brasilia Hospital.

Extraction and Protein Isolation

Soft tissues of marine invertebrates were ground with an extraction solution consisting in acetic acid 30% (v/v) in water. The suspension was centrifuged at 8,000×*g* for 30 min at 4°C. Supernatant was further precipitated with 100% $(NH_4)_2SO_4$ saturation, centrifuged at 8,000×*g* for 40 min, and extensively dialyzed with distilled water using membranes (MW cut off 3.0 kDa) (Spectrapore, USA). Subsequently, the samples were lyophilized and resuspended in 0.1% trifluoroacetic acid (TFA). The sample was submitted to high-performance liquid chromatography (HPLC) (Vydac) using a C_{18} column (Hesperia, CA, USA) system and a linear acetonitrile gradient 5-95% in 0.1% TFA at a flow rate of 0.8 mL min^{-1}. Purified fractions were lyophilized and later resuspended in phosphate-buffered saline (1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 154 mM NaCl, pH 7.4) (PBS). The protein quantification was performed by using Quant-iT™ Protein Assay Kit (Invitrogen, USA) according to the manufacturer's recommendations.

SDS-PAGE Analyses

Molecular mass analyses were conducted by SDS-PAGE as described by Laemmli [16]. Gels were stained with Coomassie Brilliant blue G-250 (Sigma, USA). Sigma Marker wide range (6.5–200 kDa) was used as standard.

Antibacterial Bioassays

The antimicrobial bioassays were carried out by microspectrophotometry (Biotek, USA) using 96-well microplates according to Hetru and Bulet [14]. Bioassay analyses against bacteria were performed in LB broth (Luria–Bertani, pH 7.0). Previously, a growth curve of the original culture was established in order to determine the relation between colony forming units (CFU) and optical density. For antimicrobial activity evaluation, 0.1 mL of inoculum was cultured in 4 mL LB medium for about 3 h until reaching the mid-logarithmic-phase. After that time, an aliquot corresponding to 5×10^5 CFU mL^{-1} and 100 μg mL^{-1} of protein from crude extracts or purification fractions was added to a solution containing LB medium to produce a final volume of 0.1 mL in the microplate wells. The microplates were incubated at 37°C and bacterial growth was monitored at 620 nm every 30 min until the bacterial growth reached the stationary phase. Chloramphenicol at a concentration of 40 μg mL^{-1} was used as positive control and distilled water and PBS were used as negative controls. All bioassays were performed in triplicate.

Amino acid Sequencing and In Silico Analysis

The N-terminal amino acid sequence of the purified peptide was determined on a Shimadzu PPSQ-23A automated protein sequencer performing Edman degradation. PHT-amino acids were detected at 269 nm after separation on a reversed-phase C18 column (4.6 × 2.5 mm^2) (GE Healthcare, USA) under isocratic conditions, according to the manufacturer's instructions. The amino acid sequence was compared to the non-redundant protein database (NCBI), SWISSPROT Data Bank, and the antimicrobial peptides database (APD) using BLAST2 Program [18].

Statistical Analysis

Inhibition percentages were transformed by ArcSin SQRT for a subsequent one-way analysis of variance (one-way ANOVA). Normality and variance homogeneity were assessed by Kolmogorov–Smirnoff and Bartlett tests,

Table 1 Antibacterial activities of different Caribbean Sea invertebrate crude extracts collected from the northern coast near Havana

Species	Phylum	*Escherichia coli* inhibition (%)	*Staphylococcus aureus* inhibition (%)
Budonosoma granulifera	Cnidaria	75.5 ± 1.4	64.9 ± 2.6
Cassiopeia xamachana	Cnidaria	78.5 ± 2.8	67.1 ± 1.9
Cenchritis muricatus	Mollusca	95.9 ± 2.3	100
Chloeia fusca	Echiura	5.0 ± 1.2	0
Condylactis gigantea	Cnidaria	0	0
Echinaster echinophorus	Echinodermata	78.2 ± 2.6	90.2 ± 1.9
Eunicea calyculata	Cnidaria	100	97.4 ± 3.6
Holothuria mexicana	Echinodermata	95.7 ± 4.1	98.3 ± 1.6
Lissodendoryx isodictyalis	Porifera	0	0
Ophiocoma echinata	Echinodermata	78.0 ± 4.0	90.0 ± 2.9
Palythoa caribaeorum	Cnidaria	97.7 ± 1.5	100.0
Phallusia nigra	Chordata	22.2 ± 3.7	91.2 ± 4.3
Plexaurella dichotoma	Cnidaria	68.0 ± 3.2	66.5 ± 1.3
Xetosporangia muta	Porifera	0	0
Zoobotryum vesiculariidae	Bryozoa	0	0

Each extract was evaluated in triplicate at a standard concentration of 100 μg mL^{-1}. Values were represented by means ± standard deviation. Chloramphenicol (40 μg mL^{-1}) and PBS were used as positive and negative controls, respectively

respectively. The Tukey test was used as a post analysis ($p < 0.05$).

Results and Discussion

After collection, soft tissues of the marine, being further dialyzed, and lyophilized in order to avoid interference with bioassays. Ten out of 15 crude extracts analyzed showed growth inhibition against Gram-positive or -negative bacteria (Table 1). Only one out of 10 extracts with antibacterial activity (*P. nigra*) showed some degree of selectivity for Gram-positive bacteria. Although few antimicrobial molecules from chordates have been evaluated as antimicrobials, almost all of them have a wide spectrum of activity [11, 17]. Only small peptides containing dehydrodopa-derived units, that have been identified in several species of tunicates, such as *Scidia nigra,* have exhibited bactericidal activity toward Gram-negative bacteria, but were incapable of controlling Gram-positive bacteria growth [21]. The highest inhibitions against bacteria were achieved using extracts from the mollusk *C. muricatus* and cnidarians *E. calyculata* and *P. caribaeorum*. *Cenchritis*

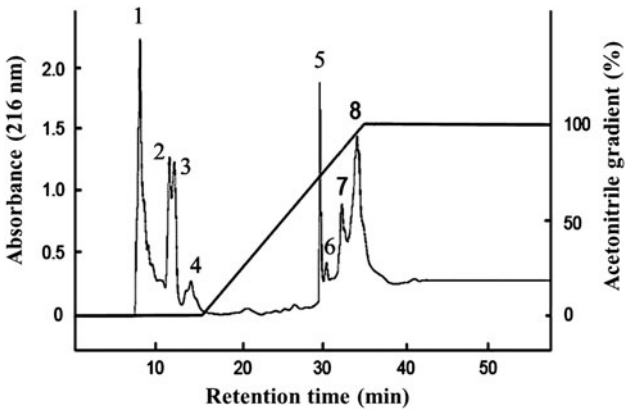

Fig. 1 HPLC reversed-phase chromatogram profile (Vydac C18-TP) of *Cenchritis muricatus* snail crude extract (1 mg). The *diagonal line* indicates a linear acetonitrile gradient

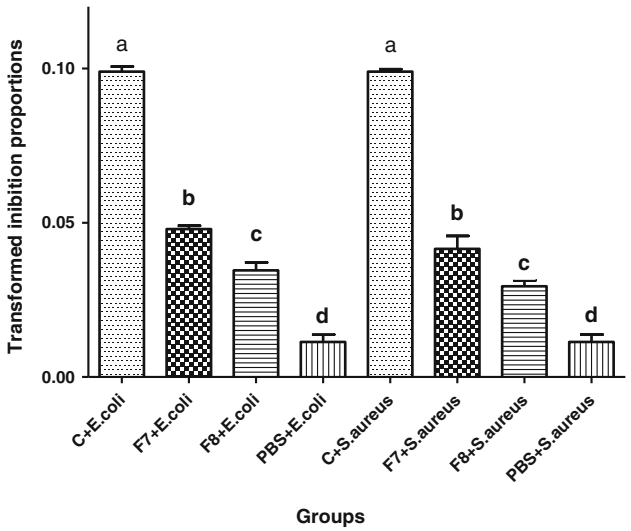

Fig. 2 Inhibitory activities of reverse phase HPLC fractions (F7, F8) against *E. coli* and *S. aureus*. Each group was evaluated in triplicate at a standard concentration of 100 μg mL^{-1}. As controls, PBS and chloramphenicol (40 μg mL^{-1}) in LB medium were used. Values represent the means calculated by ArcSin SQRT (inhibitory percentage). *Dispersion bars* represent standard deviation. One-way ANOVA was used as a means significance test. *Different letters* indicate significant differences in a post test (Tukey, $p < 0.05$)

Table 2 Antibacterial activities of semi-purified HPLC fractions from *C. muricatus* crude extract

Groups	*E. coli* growth inhibition %	*S. aureus* growth inhibition %
Chloramphenicol	94–100	96–99
F-7	22–24	14–21
F-8	10–13	8–10
PBS	1–2	1–2

Each group was evaluated in triplicate at a standard concentration of 100 μg mL^{-1}. Extreme range values of percentage are presented. Chloramphenicol (40 μg mL^{-1}) and PBS were used as positive and negative controls, respectively

muricatus is a marine mollusk that lives on seacoast rocks above the high tide line, proving to be much easier to collect than cnidarians. In this view, the ease of collection and higher activities lead us to focus on this specific specie.

To isolate the antibacterial molecule, the extract from *C. muricatus* was applied to an analytical RP-HPLC C18. Eight fractions were achieved by a chromatographic purification process (Fig. 1). An antimicrobial bioassay was performed with all lyophilized fractions resuspended in PBS, but only fractions 7 and 8 showed positive activity toward *E. coli* and *S. aureus* (Fig. 2). The growth inhibition of both fractions was lower than the inhibition obtained from the crude extracts (Table 2). This possibly occurs because of the presence of non-peptidic antimicrobial activity in the crude extract that was eliminated in the purification process [17].

Both fractions were analyzed by SDS-PAGE (acrylamide 15%). Fraction 7 showed the presence of only two major bands with approximately 10 and 15 kDa. The 10 kDa band was highly represented. On the contrary, fraction 8 showed poor purification levels, displaying numerous bands (Fig. 3a).

Although AMPs from mollusks have been extensively studied, most of the research has focused on species such as *Mytilus edulis* and *Mytilus galloprovincialis*. These species are capable of synthesizing defensins, mytilins, and the antifungal peptide mytimycin, all of which having a molecular weight below 10 kDa [21]. Only one antimicrobial peptide from mollusks with molecular mass approximately 10 kDa has been reported. This is the case of the big defensin identified from the bay scallop *Argopecten irradians* [29]. In contrast, it was extremely common to find antimicrobial proteins in crustacean species. The crustin family consists of antibacterial proteins (10–12 kDa) isolated from crustacean haemocytes [5, 25]. Also, other antibacterial proteins have been reported in crustaceans, such as anti-lipopolysaccharide factor isolated from the swimming crab *Portunus trituberculatus* [28] and homarin: a multimeric peptide from the American lobster *Homarus americanus* [6].

To characterize both bands from fraction 7, we proceeded to sequence the N-terminal region. Two different N-terminal sequences of 19 and 20 amino acids were obtained (Fig. 3b). Both sequences have a negative net charge and are hydrophilic, features that usually AMPs do not share. Unfortunately, neither sequence showed identity with different databanks (non-redundant protein database (NCBI), SWISSPROT Data Bank, and the APD). Moreover, after searching Prosite, it was not possible to find any domain that could be formed by these sequences. The lack of in silico data could be an effect of the sequence length and/or the poor genomic and proteomic data available for many marine invertebrates. For example, at present only two peptides have been isolated from *C. muricatus* [12].

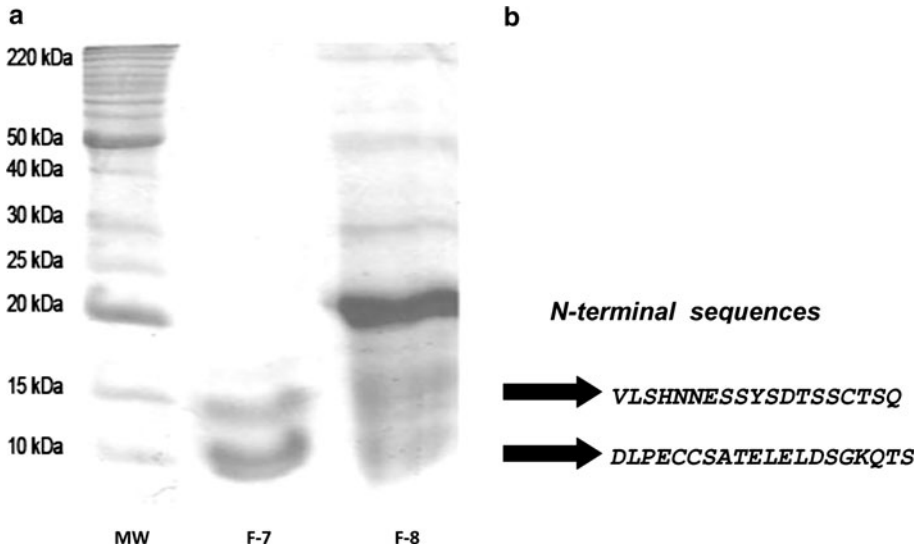

Fig. 3 **a** SDS-PAGE (15%) analysis of two semi-purified fractions using reversed-phase HPLC. *Lanes 1* Molecular weight marker, *2* Fraction 7, *3* Fraction 8. **b** N-terminal sequences of two bands of fraction 7 determined by Edman methodology

In summary, data reported here confirm the crucial importance of marine invertebrates regarding the development of newer antimicrobial therapies. In fact, 10 out of 15 species studied showed antibacterial activity. These types of proteins usually are not identified in mollusks [21], but it could have an important role in defense, as in the case of crustaceans [23]. Even in mammals that show a developed adaptive immune system, the antimicrobial proteins play a prominent role in defense [1, 2, 9]. Moreover, when further experiments are conducted for the elucidation of the antibacterial domain, these proteins could improve the knowledge about mollusk defense mechanisms; accelerating the introduction of new antimicrobial drugs for biomedical and aquacultural industries.

Acknowledgment This study was supported by CAPES, CNPq, FAPDF, and UCB.

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