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RESEARCH ARTICLE

Gill tissues of the mussel *Mytilus edulis chilensis*: A new source for antimicrobial peptides

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Abbreviations: ACN: acetonitrile
AE: acid extract
AMPs: antimicrobial peptides
BCA: bicinchoninic acid
OD: optical density
SE: Sulfoethyl

Antimicrobial peptides are small-sized, cationic and amphipathic molecules able to neutralize pathogenic microorganisms. Their antimicrobial effects tie them to mechanisms of immune defense, which is why they have been normally purified from immune cells. We describe an apparently new group of antimicrobial peptides from gill tissues of the mussel *Mytilus edulis chilensis*. 20 specimens yielded 40 g of gills which produced 16 mg of an enriched fraction with antimicrobial activity as low as 0.045 µg/µl over reference strains. Considering the chemical nature of these molecules we used an acid extraction procedure followed by consecutive cationic exchange and hydrophobic interaction chromatography steps for peptide enrichment. The resulting post Sep-pak C-18® 20% acetonitrile (ACN) eluate was fractionated by reverse phase HPLC and all resulting fractions were the source for *in vitro* antimicrobial activity evaluation. Active fractions were characterized by SDS-containing protein gel electrophoresis. All fractions were particularly enriched with low molecular

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weight peptides displaying neutralizing growth activity against Gram positive and Gram negative bacteria and 10 times more efficient over fungal pathogens. Active fractions resulted to be thermostable and non cytotoxic to eukaryotic cells. Considering these results, industrial waste gills of bivalves arise as a new source for antimicrobial molecules.

Antimicrobial peptides (AMPs) - also known as host defense peptides (HDP) are ubiquitous molecules involved in natural defense mechanisms against pathogens. They are widely distributed in animal and plant kingdoms suggesting their participation in the evolutionary success of target organisms (Zasloff, 2002). These molecules have been investigated for at least the last four decades, resulting up to the description of over a thousand of AMPs (http://www.bbcm.univ.trieste.it/~tossi/antimic.html; http://aps.unmc.edu/AP/main.php).

In invertebrates, AMPs are recognized as important components of innate immunity. They are usually low molecular weight molecules, among 12 and 45 amino acids in length, with β-folded or α - structure, cationic and amphipathic (Bulet et al. 2004). Although recent studies show that some of the peptides may have intracellular targets (Cudic and Otvos, 2002), the vast majority appear to act by permeabilization of the bacterial cell membrane (Yeaman and Yount, 2003). Some kind of peptides have disulfide bridges that determine the secondary structure, making them suitable for its antimicrobial action (Hancock and Diamond, 2000; Marshall and Arenas, 2003). In marine invertebrates, *Mytilus galloprovincialis* and *Mytilus edulis* have been the most studied (Tincu and Taylor, 2004); particularly the hemocytes, where almost all AMPs described belong to the defensin family (Charlet et al. 1996; Mitta et al. 1999a; Mitta et al. 1999b). On the other hand, epithelial tissues, as one of the first physical barrier encounter by any pathogen, have been also the centre of attention in the search for new AMPs (Iijima et al. 2003). The same has been shown for insects with the demonstration of specific AMP mRNA expression in epithelial cells (Ferrandon et al. 1998), strengthening the idea that these tissues are predominant reservoirs functioning in local defence against pathogenic microorganisms. As an example and centring on marine environments, branchial, intestinal, and epidermal tissues of fish have provided a great variety of these molecules (Richards et al. 2001; Lauth et al. 2002; Iijima et al. 2003). In bivalves, the same has been achieved for mussels (Haug et al. 2004), turning epithelial cells from the Chilean native mussel *Mytilus edulis chilensis* into a target model to screen for new and ideally novel AMPs for biotechnological purposes (Marshall and Arenas, 2003). In this report we describe an efficient recovery of at least one putatively new cationic and amphipatic AMP species displaying a broad antimicrobial range and high specific activity, thus suitable for biotechnological processing.

MATERIALS AND METHODS

Mussels and gills collection

Live specimens of *Mytilus edulis chilensis* were obtained commercially from Puerto Montt, Chile. Mussels were kept on ice until gill tissue isolation and once filaments were dissected and dried with Watman 3M paper, they were stored at -80ºC until use.

Processing gills

The purification procedure is summarized as follows. Frozen gills from 20 specimens were crushed to homogeneity in liquid nitrogen. The resulting gill powder (40 g) was suspended in cold acetic acid 11% (1:10), in order to solubilize cationic molecules, followed by vortex-mixing for 5 min. The homogenate was sonicated for 3 x 30 sec at 11 resonant macrosonic synthesis (RMS) in ice and incubated with mild agitation at 4ºC overnight. The crude extract was centrifuged at 11,000 x g, 35 min at 4ºC and the pellet was discarded. The supernatant was called acid extract (AE) and further shaken at 37ºC for 1 hr to favour sugar hydrolysis. Tris-base crystals were slowly added to raise the pH to a value of 3.0. In order to enrich cationic peptides, four batches of 100 ml each of AE were sequentially mixed overnight with a Sulfoethyl (SE) Sephadex C-50 cation-exchange beads (BioRad) with mild agitation at 4ºC in a ratio of 1,5 ml gel slurry/25 ml AE. The mixture was centrifuged at 1,500 x g 10 min and resuspended in 1% acetic acid 0.1 M NaCl (pH 3.0).

Table 1. Total protein concentration on each resulting fraction from the purification procedure.

Samples	[mg/ml]	V (ml)	Total protein [mg]	% From initial
Purified gills (40 g)				
Acid Extract	3.36	400.0	1,346.40	-----
Eluted SE fraction	3.78	12.0	55.00	4.09
Eluted Sep-pak C-18 ®				
5% ACN	0.88	0.4	0.35	0.02
20% CAN*	40.00	0.4	16.00	1.18
40% ACN	89.00	0.4	35.60	2.64
60% ACN	3.56	0.4	1.42	0.10
80% ACN	0.625	0.4	0.25	0.18

*The 20 % ACN fraction was further processed through RP-HPLC.

Putative peptides were recovered from beads with mild agitation for 1 hr in the presence of 1% acetic acid 1M NaCl (pH 3.0). The eluate (6 ml) was applied onto a Sep-pak C18 Vac cartridge (Waters Associates) equilibrated in acidified water (0.05% trifluoroacetic acid in UPW-Ultra Pure Water). After a wash with acidified water, the peptides were eluted, flow 1 ml/min, with 5%, 20%, 40%, 60% and 80% ACN, to obtain several hydrophobic fractions. The samples obtained were lyophilized and reconstituted in 200 µl of UPW, total protein content determined by the BCA microplate assay (Pierce) and tested for antibacterial activity.

HPLC purification

All HPLC purification steps were performed on a RP-HPLC model LaChrom D-7000 with a LaChrom model L-7455 photodiode array detector. Column effluent was monitored by UV absorption at 225 nm. Only the 20% Sep-pak eluates were selected for further purification and loaded on a Sephasil C-18 (250 x 4.1 mm) column (LiChroCART). Elution was performed with a linear gradient of 5-60% ACN in acidified water over 90 min at a flow rate of 0.6 ml x min^{-1}. The resulting fractions were collected every two minutes, lyophilized, reconstituted in UPW and frozen at -20ºC until antimicrobial activity testing.

Reference pathogenic strains and growth conditions

Micrococcus luteus (ATCC 4698) and *Staphylococcus epidermidis* (ATCC 49461) were used as reference Gram-positive bacteria; *Escherichia coli* (ATCC 11303) and *Aeromona hydrophila* (ATCC 23213) as Gram-negative; while filamentous fungi were represented by *Fusarium oxysporum* and *Neurospora crassa*, kindly provided by Dr E. Bachère from Université Montpellier-2**,** France. The aquatic fungi *Saprolegnia* sp. was a generous gift from Dr. Piontelli - Universidad de Valparaíso, Chile. All bacteria were grown at 37ºC in Trypticase Soy Broth (TSB, Difco), *F. oxysporum* and *N. crassa* were grown at room temperature in Potato Dextrose Broth (PDB, Difco) and *Saprolegnia* sp. was grown in Glucose Yeast (GY) broth. All fungal spores were harvested as described previously (Broekaert et al. 1990).

Antimicrobial activity assay

Antibacterial activity of all resulting fractions was determined by the microplate assay described by Mitta et al. (1999). *M. luteus*, *A. hydrophila*, *S. epidermidis* and *E. coli* were incubated at 37ºC in TSB until reaching an optical density (OD) 0.2 - 0.3 at 620 nm. Antibacterial activity was determined by incubating standard aliquots of 100 µl of a 1/100 dilution of the bacterial solution in triplicates, exposed to 10 µl of target peptides. After 24 hrs incubation at 37ºC, absorbance values at 620 nm were determined. Percentage growth inhibition was determined by subtracting bacterial growth values in the absence of peptides from that displayed by bacteria exposed to different concentrations of the target molecules. As a positive control for growth inhibition comercial cecropin P1a (SIGMA), was used at a concentration of 0.01 µg/µl amount known to fully inhibit Gram negative bacterial growth.

Antifungal activity was monitored against *F. oxysporum, N. crassa* and *Saprolegnia* sp. by a liquid growth inhibition assay, as described by Fehlbaum et al.(1994) with minor changes. In summary, 80 µl of fungal spores to a final concentration of 10^4 spores x ml^{-1}, were added to 20 µl of peptide solution on microtiter plates in the presence of tetracycline (10 µg/ml) and streptomycine (10 µg/ml), followed by 30 min incubation at room temperature after which absorbance at 595 nm was determined. The plates were further incubated under light at room temperature for another 72 hrs and final OD values at 595 determined. Growth inhibition was established by subtracting fungal growth values at 72.5 hrs in the absence of peptides from that displayed by fungal spores exposed to different concentrations of the target molecules.

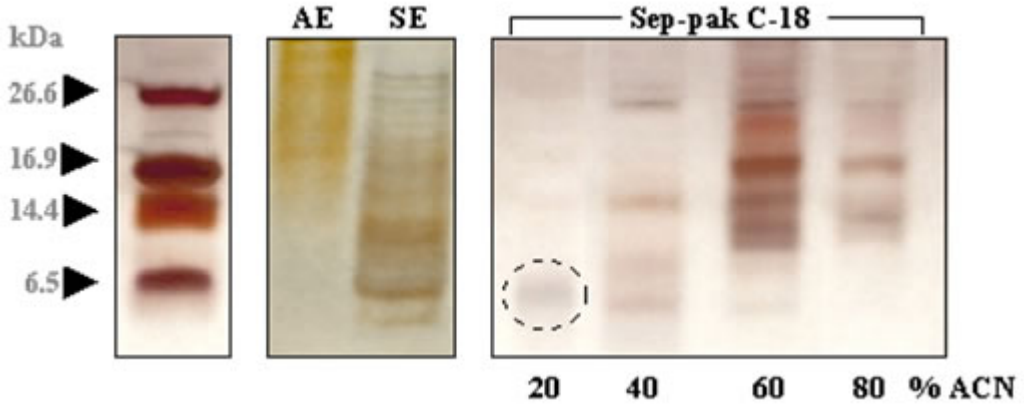

Figure 1. Electrophoretic profiles of low molecular weight enrichment of AMPs during gill processing. Identical concentration aliquots resolved through 18% Tris-ricine SDS/PAGE. Silver Nitrate stained gels. AE, acid extract; SE, sulpho-ethyl Sephadex elution; Sep-pak C-18; differential elution fractions.

Cytotoxicity assay

The effect of active fractions over the viability of eukaryotic cells was measured by exposing an established Chinook salmon embryo cells (CHSE-214) according standard procedures developed in our laboratory. Briefly, cell monolayers at 70% semiconfluency were washed with PBS and then the peptides added at a concentration of 1.5 µg/µl per triplicate wells and incubated for the maximum viability time (3 hrs) without culture medium. Samples were then washed 3 times with excess PBS before adding 0.1% trypsin to release cells from the monolayer. Individual cell viability was determined using the Trypan Blue exclusion technique (Lee et al. 2002).

Heat treatment

The 20% ACN peptide-enriched fraction was tested for heat sensitivity. Two parallel samples were treated either at 50ºC or 100ºC for 30 and 60 min, respectively. Immediately thereafter, their antibacterial activity was tested against *M. luteus* using non-heated samples as controls.

Electrophoresis

To estimate low molecular weight peptide sizes, 18% denaturanting SDS-PAGE, polyacrylamide tris-tricine-urea gel electrophoresis was employed (Schagger and von Jagow, 1987). In order to calculate relative molecular weights, Polypeptide SDS-PAGE commercial standard marker was used (BioRad). Gels were stained either with Coomassie brilliant blue and/or Silver Nitrate kits (Winkler) depending on protein concentration in samples.

Table 2. Citotoxicity assay of the 20% ACN fraction and post RP-HPLC active fractions over CHSE-214 cells.

Samples assayed for cytotoxicity	% viability
20% ACN	77.9
post HPLC	
fx 19	73.0
fx 20	82.1
fx 21	84.4
fx 22	86.6
Distilled water (blank)	77.2

RESULTS

Table 1 shows a comparative protein concentration recovery from gill samples enriched in active AMPs throughout the process described in this report. From the selected enriched fraction (post RP-HPLC 20% ACN) yield equals 1.18% of low molecular weight AMPs per 40 g of processed *Mytilus edulis chilensis* gills.

Figure 1 shows the polypeptide profiles throughout the processing procedure. Compared to AE, SE and post Sep-pak 20% and 40% ACN fractions are clearly enriched in low molecular weight peptides. Nonetheless, the content of the 20% fraction is particularly enriched in low molecular weight peptides. Although the 40% fraction is more heterogeneous but also seems enriched in these low molecular weight species, the specific activity of the former as well as their uniqueness drove us to favour their characterization.

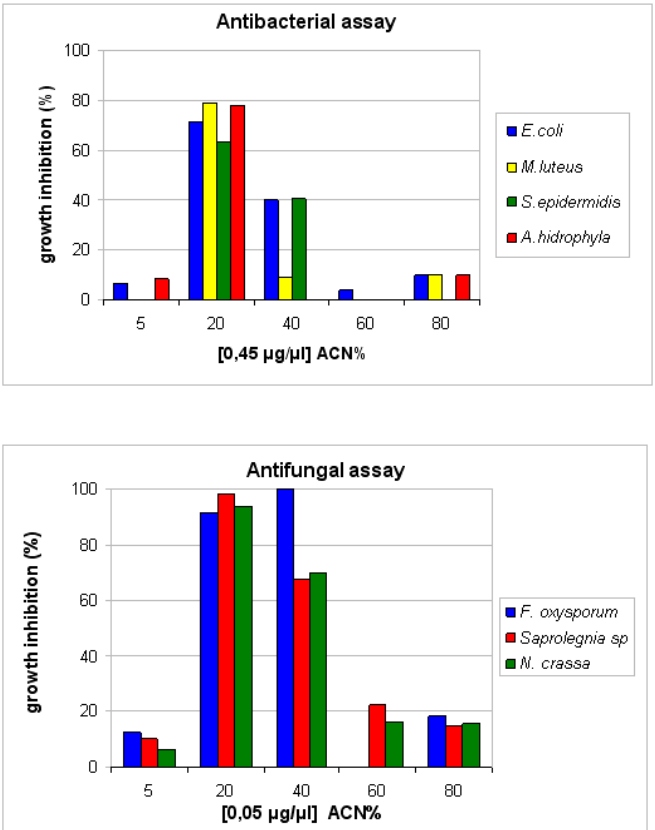

Figure 2. Antibacterial and antifungal activity of 5 to 80% ACN post Sep-pak eluates.

Figure 2 shows the results of all post Sep-Pak C-18 fractions tested for antimicrobial activity over standard strains of bacteria (G+ and G-) as well as over selected fungi strains. In general, testing required 10 higher concentrations for bacteria than for fungi. For bacteria, the 20% ACN fraction was the most efficient with a minimal growth inhibition of 65% although inhibition over 75% was obtained over *M. luteus* and *A. hydrophila*. For fungi, both fractions 20% and 40% ACN were equally efficient, also with a minimal growth inhibition of 65%. Noticeably, inhibition reached over 90% for the 40% ACN fraction over *Fusarium oxysporum*, but the 20% ACN fraction was by all means more effective over most of the fungal strains tested reaching inhibition values close to 90%.

In order to evaluate another physicochemical parameter associated with these novel molecules we determined if heat had any effect over the observed antimicrobial activity. Aliquots of the 20% ACN fraction were heated at either 50ºC or 100ºC for 30 or 60 min. Figure 3 shows that using *M. luteus* as a reference strain, the activity was not at all affected at a concentration of 0.45 µg/µl.

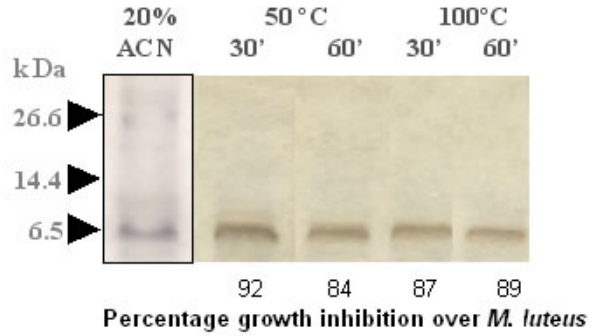

Figure 3. Thermal effect over the structure and antimicrobial activity of the post-Sep-pak C-18 20% ACN fraction. Tris Tricine 18% SDS/PAGE.

Next, to further resolve the 20% ACN fraction, 300 µg were loaded onto an RP-HPLC. Figure 4 shows all resulting fractions of which only numbers 19 to 22 (12-15 µg each) eluting between 26-30% ACN were active against *M. luteus.* Resolution through 18% tris-tricine SDS-PAGE (insert in Figure 4), confirms a clear enrichment in a low molecular weight band over the control.

Finally, in order to rule out cytotoxicity of the 20% post sep-pak eluate as well as the post RP-HPLC positive fractions, 1,5 µg/µl in distilled water of each, corresponding to three times the inhibitory concentration over the reference bacterial strain were exposed for 3 hrs to the fish cell line CHSE-214 (Table 2). None of the fractions tested affected cell viability.

DISCUSSION

AMPs from marine invertebrates have been normally isolated from hemocytes (Patrzykat and Douglas, 2003). When reported for epithelial tissues it has been assumed that it was due to hemocyte infiltration (Mitta et al. 2000). Nonetheless, a recent report describes antimicrobial activity associated with crude extract from different tissues obtained from the mussel *Modiolus modiolus* (Haug et al. 2004), although the activity was not linked to low molecular weight molecules. In this report the amount of peptides recovered from isolated gills suggest that most likely gills, and perhaps other epithelial tissues, naturally express endogenous AMPs. Mussel hemocytes AMPs are normally isolated after an acid extraction step followed by a Sep-pak column enrichment in which peptides are eluted

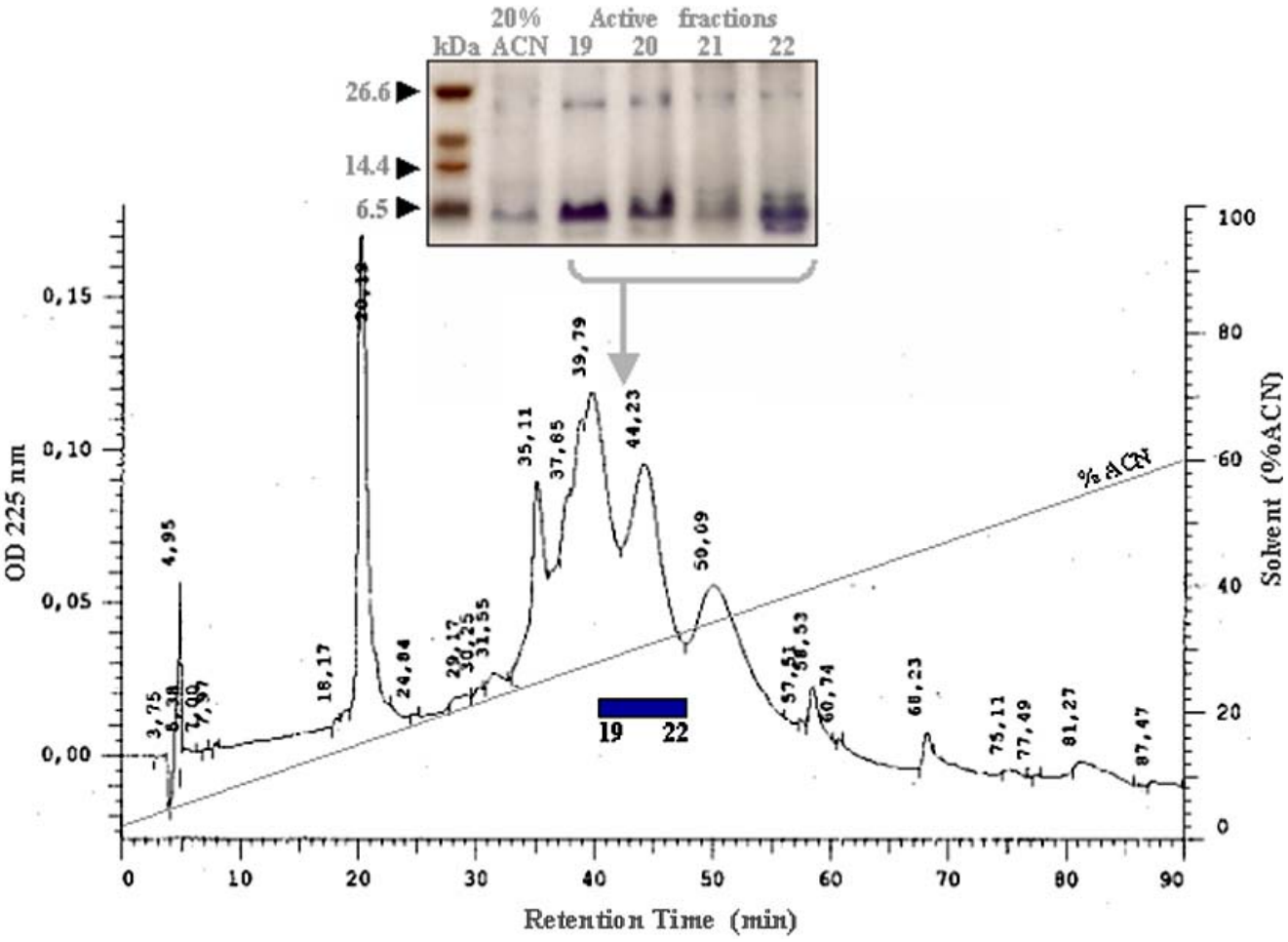

Figure 4. RP-HPLC elution profile of the 20% ACN post Sep-pak C-18 with a gradient of 5 - 60% ACN in 0.05% TFA. Bar: active fractions. Insert: Tris-Tricine 18% SDS-PAGE characterization of active fractions.

with step gradients of 5 - 40 - 80% ACN, and active peptides are mostly recovered in the 40% fraction. For gills, the step gradient elution procedure was widened including two additional steps of 20 and 60% ACN. Although gills also provided a 40% AMP rich fraction, we found an as yet unreported noticeably antimicrobial activity in the 20% ACN elution fraction. These results suggest that this fraction is enriched in a less hydrophobic thermostable distinct class of active AMPs which displays a powerful action over fungi as well as both Gram - and Gram + bacteria. Thus, we decided to further characterize the novel 20% ACN fraction through RP-HPLC recovering active molecules using a linear 5 - 60% ACN elution gradient. Out of 45 total fractions, active AMPs eluted in four distinct fractions between 26 and 30% ACN devoided of cell cytotoxicity.

Based upon our results and considering that mussel gills represent roughly 13% of the smooth body weight portion, we consider this tissue as a reasonable source for new antimicrobial molecules. If this is the case, this approach could be extended in the future to other tissues, especially to those involving tissues discarded in the industrial processing of commercial marine invertebrates. Thus, we have demonstrated in this report that adding a slight modifications to existing protocols broadened the range of extractable AMPs resulting in high specific activity new species in the 20% ACN fraction. Additionally we have foresee a potential use for byproducts in the aquaculture industry as a source for new pharmaceutical molecules. We are in the process of designing strategies to prove the feasibility of these alternatives.

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