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RESEARCH REPORT

First evidence of antimicrobial activity of neurotoxin 2 from *Anemonia sulcata* (Cnidaria)

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Abstract

We investigated the antibacterial activity of *Anemonia sulcata* (Cnidaria, Anthozoa) tentacle and body acidic extracts. Biochemical purification consisted of first step on solid phase Sep-Pak C8 column followed by several HPLC runs on C18 column using different conditions. Anti-*Micrococcus lysodeikticus* activity has been detected in 40 % acetonitrile fractions. The resulting purified molecule from tentacles had a molecular mass determined by MALDI-TOF mass spectrum of 4946,299 Da and has been completely sequenced. Its aa sequence revealed identity with the Neurotoxin 2 (ATX-II), a Na^+ channel blocking toxins. Consequently, ATX-II appeared to display a dual role as toxin and as antibacterial.

Key Words: antimicrobial peptide; *Anemonia sulcata;* ATX II; neurotoxin; *Micrococcus lysodeikticus*

Introduction

The sea anemone *Anemonia sulcata* is a widespread Mediterranean species. Sea anemones are generally poisonous animals that spend most of their lives in a sessile form. Capturing activities and defense mechanisms are strongly associated with toxin production (Ruppert and Barnes, 1994). The neurotoxin ATX II is a type 1 sodium channel toxin and consists of 47 amino acid residues, linked by three disulfide bridges. ATX II specifically binds to the sodium channel (site 3), thus delaying its inactivation during signal transduction; has a strong effect on crustaceans and insects and a weaker effect on mammals. These toxins specific for sodium channels have been thoroughly investigated also because they constitute a major fraction of the venom (Moran *et al.,* 2009).

Antimicrobial peptides are important defense molecules in marine invertebrates covering a broad-spectrum of bacteria and fungi (Boman, 2003; Bulet *et al.,* 2004). They are defined as molecules of less than 10 kDa characterized by immediate and rapid response to invading microorganisms (Bartlett *et al.,* 2002). There is evidence that antimicrobial peptides are widespread in invertebrates (Chisholm and Smith, 1992), especially in tissues such as the gut and respiratory organs in marine invertebrates, subjected to a first exposure to pathogens. In spite of variations in structure and size, most of antimicrobial peptides are amphiphilic, displaying both hydrophilic and hydrophobic surfaces (Tincu *et al*., 2004). AMP are exciting candidates as new antibacterial agents due to their broad antimicrobial spectra, highly selective toxicities, and the difficulty for bacteria to develop resistance to these peptides (Boman, 1998; Lehrer and Ganz, 1999; Hancock *et al.,* 2000). Therefore, AMPs from marine organisms represent a largely unexploited resource that can afford design of new antibiotics with broad-spectrum antimicrobial activity (Ovchinnikova *et al.,* 2006). It is plausible that certain functional molecular motifs, such as amphipathic α-helices and β-sheets, would have featured predominantly in the arsenal employed by our distant eukaryotic ancestors. Over time, these would have diversified through mutation and selection pressure to produce the great variety of AMPs and other molecules (Smith *et al.,* 2010).

Several AMPs have been isolated from phylum of Cnidaria. Have been reported the purification of a 40-residue AMP from the mesoglea of a scyphozoid jellyfish, *Aurelia aurita* (Ovchinnikova *et al.,* 2006). The peptide was named aurelin and exhibited activity against gram-positive and gram-negative

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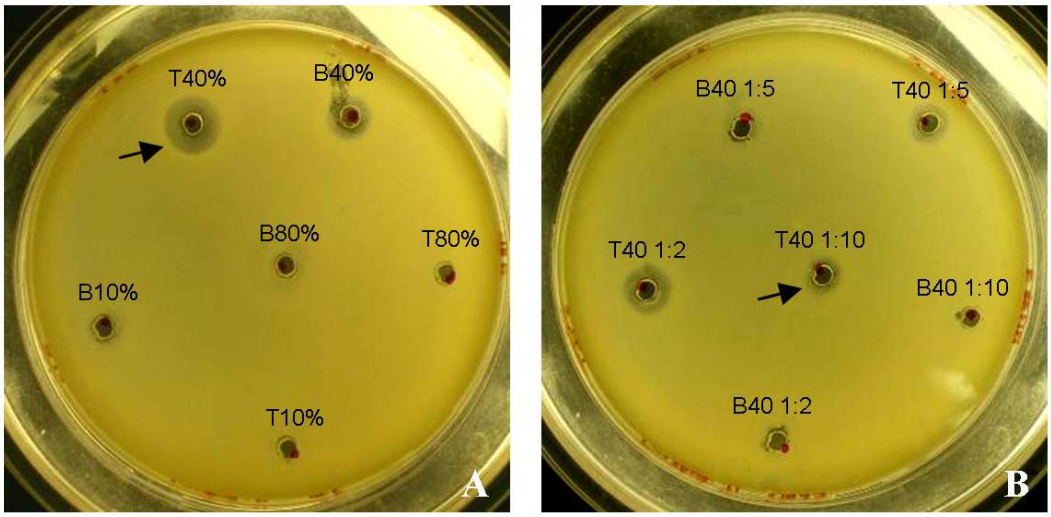

Fig. 1 Tentacles and body acid extracts of *Anemonia sulcata* were loaded onto solid phase column (Sep-Pak C_8). The resulting 10, 40 and 80 % acetonitrile fractions (10 µl) were tested for the antibacterial activity toward *Micrococcus lysodeikticus* by filling wells performed in Petri dishes containing trypticase soy agar (A). The 40 % active fraction have been also assayed at different dilution (B).

bacteria. Furthermore, a detailed biochemical characterization of the antimicrobial activity in *Hydra* revealed the presence of conserved AMPs such as Hydramacin-1 and novel taxon-specific AMPs such as Arminin 1a and Periculin-1 which have no counterpart in the transcriptomes of any other organisms (Bosch *et al*., 2009). It could be possible to obtain, from this molecule, synthetic peptides with significant antibacterial activity and relatively low cytotoxicity to human erythrocytes, as in the case of stycholysin I and II (Tejuca *et al.*, 1996), 2 pore-forming cytolysins from the sea anemone *Stychodactyla helianthus*.

In this paper we reported *i)* the presence of anti-*Micrococcus lysodeikticus* activity in acidic extracts from tentacles and body of *A. sulcata; ii)* the biochemical purification of the active peptide, and *iii)* its complete aa sequence revealing identity with Neurotoxin 2 (ATX II).

Material and Methods

Animals and tissues sampling

Anemonia sulcata (Anthozoa) adults have been collected from Termini Imerese (Palermo, Italy) and maintained in oxygenated sea water at 18 °C. Acid extractions were performed from tentacles and body. Briefly, tentacle were separated from the animal body with a forceps and both suspended in Tris buffered solution (TBS, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4). After homogenization in ice bath with Ultra-Turrax for 5 min, 10 % acetic acid was carefully added. The 2 samples have been sonicated (Branson Model B15, Danbury, CT) 3 times for 30 sec each, then centrifuged at 21,000x*g*, for 20 min at 4 °C. Protein content of both supernatants was determined using the NanoDrop ND-1000 spectrophotometer.

Antibacterial assays

Solid phase elution fractions have been freeze-dried and suspended in 50 µl sterile ultra pure water (UPW). Antibacterial activity has been tested against *M. lysodeikticus* by filling wells performed in Petri dishes containing 10 ml of trypticase soy agar (TSA) and 3 ml of 0.5 McFarland turbidity. Each well received 10 µl of sample and Petri dishes were incubated overnight at 37 °C. Diameter of clear ring around the wells paralleled the antibacterial activity. Liquid growth inhibition assay has been used to check for antibacterial activity in all HPLC fractions. Briefly, fractions were freeze-dried and suspended in 50 µl UPW. Twenty µl aliquots were added to 20 µl of a suspension of *M. lysodeikticus* ($5x10^6$ bacteria/ml) in Poor-Broth nutrient medium (1 % Bactotrypton, 0.5 % NaCl, w/v) in 96 wells microtiter plates. Bacterial growth was measured at A_{600} with a microplate reader Tecan (Infinite 200 M) during 16 h at 37°C. Minimal inhibitory concentration (MIC) was obtained by testing serial doubling dilutions of selected purified peptide in liquid growth inhibition assay as above and corresponded to the lowest concentration that caused 100 % growth inhibition. Minimal bactericidal concentration (MBC) was determined by plating the contents of the first 5 wells with no visible growth of bacteria onto TSA Petri dishes. The lowest concentration that prevented any colony formation unit (CFU) as observed after 18 h incubation at 37 °C corresponded to the MBC.

Solid phase extraction and reversed phase HPLC purification

Tentacles and body acid extracts were loaded onto Sep-Pak C_8 Vac cartridges (Waters Associates) equilibrated with acidified water (0.05 % TFA in UPW). After washing with acidified water, three successive elutions were performed

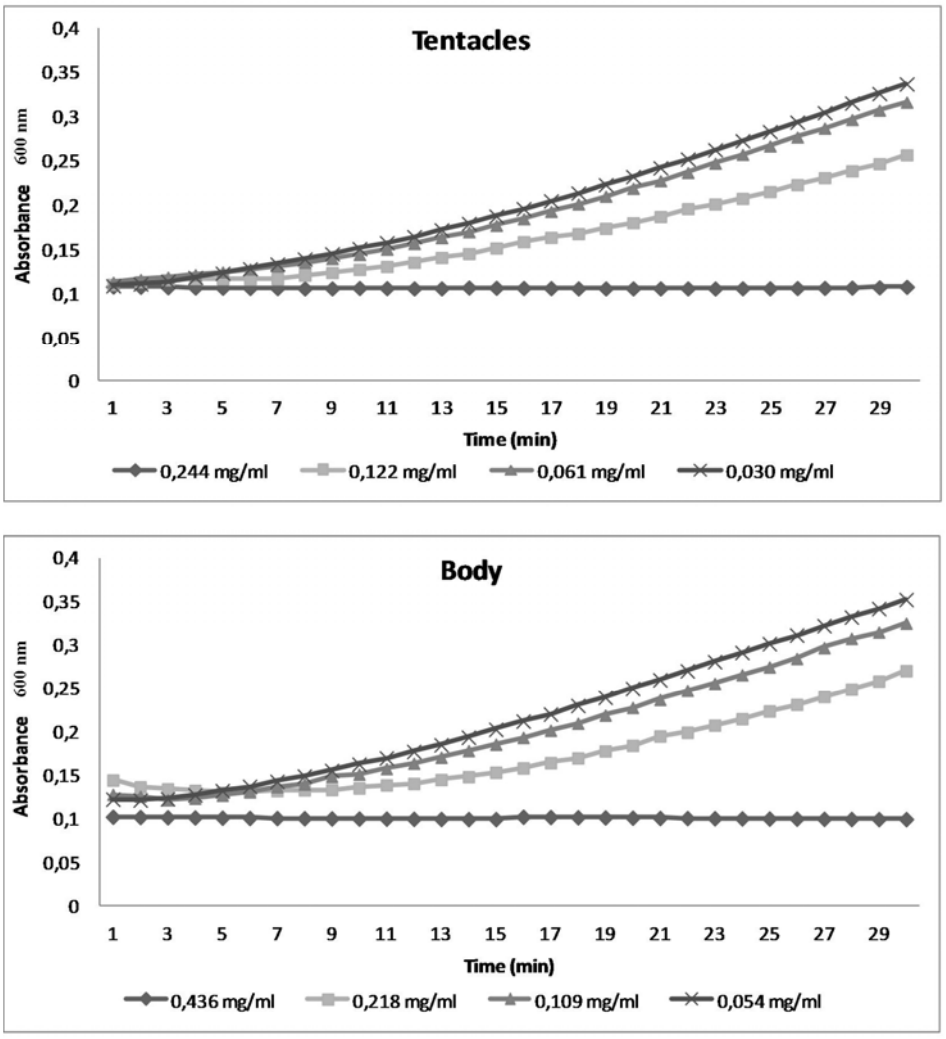

Fig. 2 Tentacles and body acid extracts of *Anemonia sulcata* were loaded onto solid phase column (Sep-Pak C_8). Minimum inhibition concentration of the 40 % active acetonitrile fraction was assayed toward *Micrococcus lysodeikticus* obtained after incubation in the microplate reader TECAN for 16 h at 37 °C. The data obtained show an antibacterial activity at concentrations up to 0.244 mg/ml.

successively with 50 ml of 10, 40 and 80 % acetonitrile in acidified water. Fractions were freeze-dried and reconstituted with UPW. As the bulk of the antimicrobial activity was detected in the 40 % fraction, only this fraction was submitted to reversed phase HPLC on a silica column C_{18} Interchrom UP5ODB-25QS (250x4.6 mm). Elution of the 50 µl sample was performed with a linear gradient of 0 - 60 % acetonitrile in acidified water over 60 min at a flow rate of 0.5 ml/min. Fractions corresponding to absorbance peaks detected at both 280 and 225 nm were collected in polypropylene tubes, freeze dried, reconstituted in UPW and tested for anti-*M. lysodeikticus* activity by the liquid growth inhibition assays described above. Active fractions were then purified on the same reversed phase column, with a linear gradient from 20 - 60 % acetonitrile in acidified water at a flow rate of 0.5 ml/min. Each collected peak was freeze-dried, reconstituted in UPW and tested for anti-*M. lysodeikticus* activity.

Mass spectrometry

The molecular mass of the active peak content was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with an Ultraflex TOF/TOF (Bruker Daltonics). The 2,5-dihydroxybenzoic acid (DHB) matrix was prepared in Acetonitrile/Ultrapure Water/TFA (20/80/0.1 %) for a final concentration of 5 mg/mL. One µL of sample dissolved in 0.1 % TFA was mixed with 1 µL of the matrix solution on the steel target and dry in ambient air. The calibration was performed with five peptide standards: angiotensin II (MH+ = 1046.54), angiotensin I (MH^+ = 1296.68), Neurotensin (MH^+ = 1672.91), ACTH clip 1-17 (MH^+ = 2093.08) and ACTH clip 18 - 39 (MH^+ = 2465.19). The data are acquiring with the flexControl software (version 3.3.108.0) in reflector mode and with an accelerating voltage in the ion source of 25 kV. Spectrum was analyzed with the flex Analysis software (version 3.3.80.0).

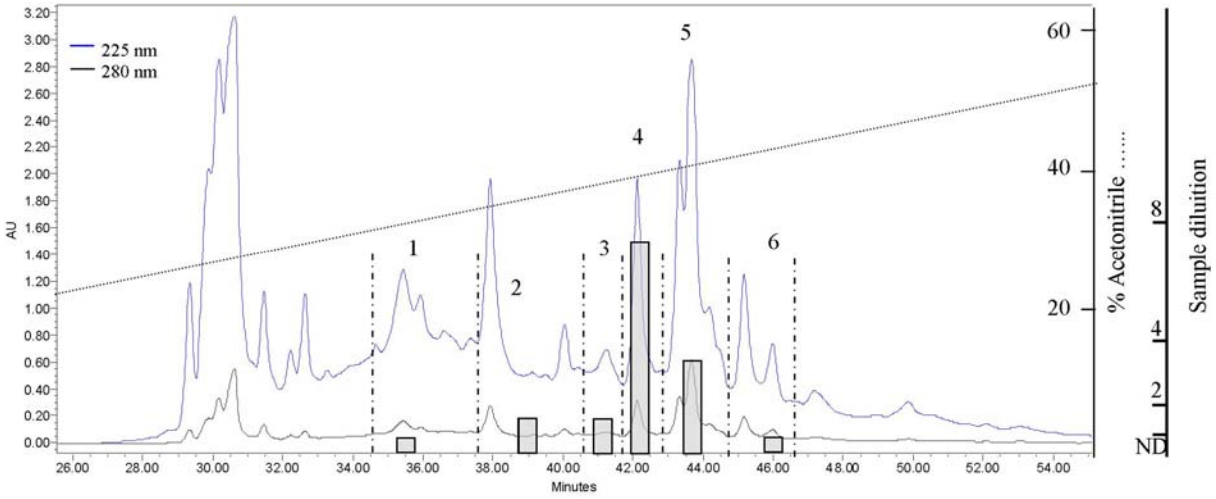

Fig. 3 Reversed phase HPLC separation of the tentacles of *Anemonia sulcata*. After pre-purification by solid phase extraction, the active material present in the fraction eluted with 40 % acetonitrile was analyzed on an silica column C18 INTERCHROM UP5ODB-25QS 250x4.6mm; with a hydrophobic chain of 18 carbon atoms column. Elution was performed with a linear gradient (dotted line) from 0 to 60 % acetonitrile in acidified water (0.05 % trifluoroacetic acid) over 60 min at a flow rate of 0.5 ml/min. Absorbance peaks were monitored at 225 and 280 nm (blue and black full line respectively). Histograms show the antibacterial activity of the peaks toward *Micrococcus lysodeikticus.*

Amino acid sequencing

The peptide is deposited on a glass fiber disc pre-coated with Biobren Plus (Applied Biosystems). The dried disc is introduce into a Procise P494 automated protein sequencer (Applied Biosystems). The amino acids are determinate by multiple cycles of automated Edman degradation coupled on-line to a microgradient system (Model 140C) and to a HPLC PTH-amino acid analyzer (model 785A). The amino acid sequence of the peptide, deduced from the chromatograms generated by the acquisition data, was compared to sequences in UniProtKB/Swiss-Prot public protein sequence databases using Blast tool from NCBI Protein Blast website.

In Silico *analysis*

The *in silico* analysis allowed to determine which of the two neurotoxins purified had higher probability of being a factor antimicrobial. The database of prediction used were: APD; The Antimicrobial Peptide Database, CAMP; Collection of Antimicrobial Peptides and AMPA; (Wang and Wang, 2004; Shaini *et al.,* 2009; Torrent *et al*., 2009, 2012). APD: The Antimicrobial Peptide Database is a site prediction based on two characteristic indices for the determination of antimicrobial power: Wimley-White hydrophobicities and Boman Index (Wang and Wang, 2004). Wimley-White hydrophobicities is a scale of hydrophobicity (Kcal/mol) which defines the relative hydrophobicity of amino acid residues, the more positive is the value of this index, the more hydrophobic amino acids are located in that region of the protein. This scale is commonly used to predict the transmembrane alpha-helices of membrane proteins (White and Wimley, 1999). The Boman Index (potential protein binding) is defined as the sum of the free energies of the side chains of amino acid residues divided by the total number of amino acid residues. A low value (≤ 1) indicates that the peptide has a higher antibacterial activity without many side effects, while a higher value (2,50 - 3,00) indicates that the peptide is multifunctional with activity similar to hormones (Boman, 2003). The possibility of an alignment (ClustalW2) for the structure homology was also verified using SWISS-MODEL.

Results

Purification of AMP from the tentacles of A. sulcata antibacterial activity of the SEP PAK products

The 10, 40 and 80 % acetonitrile (ACN) fractions of the *A. sulcata* tentacle obtained through SEP PAK were tested to evaluate the antibacterial activity. Preliminarily was determined the protein concentration of the same fractions, which showed a higher amount of protein in the fraction of 40 % ACN (7.83 ± 2 mg/ml) compared to the 10 % ACN fraction (0.98 ± 0.12 mg/ml) and a minimum amount in the protein fraction to 80 % (0.20 ± 0.09 mg/ml).

The fractions were tested against different bacterial strains, highlighting specific antibacterial activity towards *M. lysodeikticus*. The activity was found only in the fraction at 40 % acetonitrile and was highlighted significant activity up to a dilution of 1/10 (Fig. 1).

Following assays of samples on 96-well plate towards *M. lysodeikticus* and subsequent incubation in the microplate reader TECAN for 16 h at a temperature of 37 °C, the data obtained show an antibacterial activity at concentrations up to 0.244

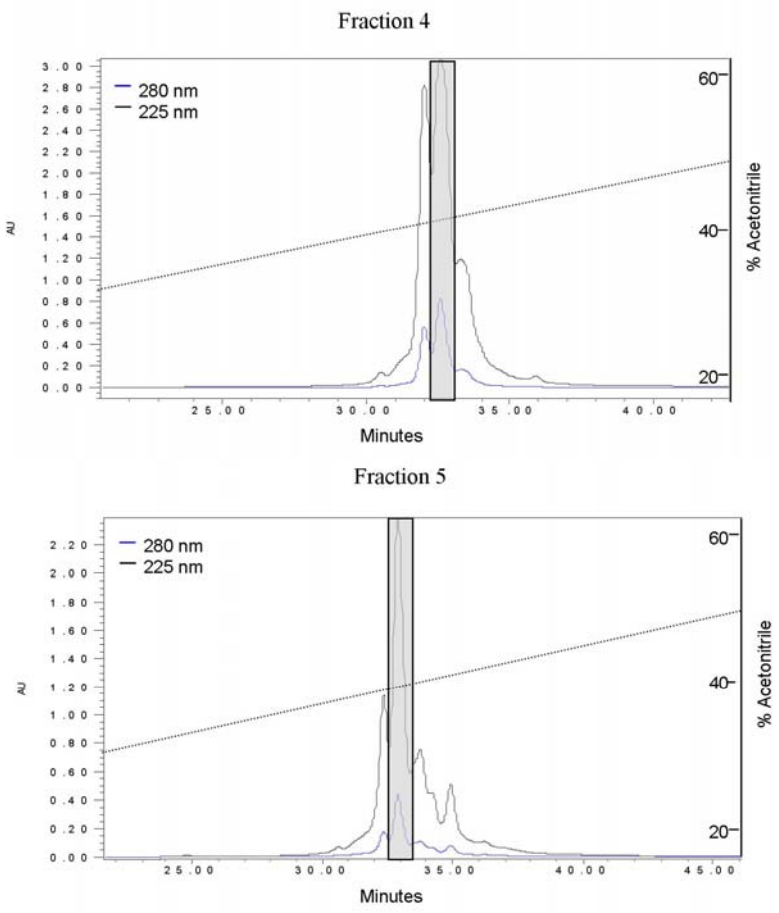

Fig. 4 Profiles of separation of the fraction 4 and fraction 5 of the tentacles of *Anemonia sulcata* obtained by reverse phase chromatography with a gradient of acetonitrile from 20 to 60 % in acidified water (0.05 % trifluoroacetic acid) over 60 min at a flow rate of 0.5 ml/min. Absorbance peaks were monitored at 225 and 280 nm (blue and black full line respectively). Histograms show peaks active toward *Micrococcus lysodeikticus.*

mg/ml for the tentacles and antibacterial activity at concentrations up 0.436 mg/ml for the body, showing a higher potency of the extract sprawling respect to the body (Fig. 2). At lower concentrations it has an exponential bacterial growth also confirmed by subsequent testing of the same samples on petri dishes.

Reverse phase chromatography

The active molecules of the 40 % eluate were submitted to reversed phase HPLC and eluted with a gradient of 0 - 60 % acetonitrile, yielding the chromatogram shown in Figure 3. The graphs show a series of peaks from 30 min of entering the sample. All peaks were collected and assayed to *M. lysodeikticus* to try to identify potential antimicrobial factors. The assays show a high antibacterial activity in peaks collected between 34 and 46 min named fraction 1, 2, 3, 4, 5 and 6 (Fig. 3).

All the fractions which show high antibacterial activity were further purified by reverse phase chromatography with a gradient of acetonitrile from 20 to 60 %. The fractions 2 and 3 purified further show no significant peaks (data not shown), on the contrary fractions 4 and 5 show several peaks and in both fractions there is a single peak showing antimicrobial activity towards *M. lysodeikticus* (Fig. 4).

Mass spectrometric analysis and sequencing

The peak of fraction 5, which is found to be the most pure, was analyzed by mass spectrometry and sequencing.

The mass spectrometric analysis showed the presence of two peptides, the first from the molecular weight of 4946,299 Da present preponderantly and second, present in traces, from the molecular weight of 4808.174 (Fig. 5).

The sequencing by the method of Edman detecting the presence of Neurotoxin 2 (ATX-II) (Wunderer *et al.,* 1976) specific for the sodium channel with theoretical molecular weight of 4945.30 Da in addition a small amount of Blood depressing substance (BDS II) (Doppelfeld *et al.,* 1985; Diochot *et al.,* 1998) specific for potassium channels by the PM of 4773.12 Da was also present (Table 1). These results are in agreement with the results of mass spectrometry weight.

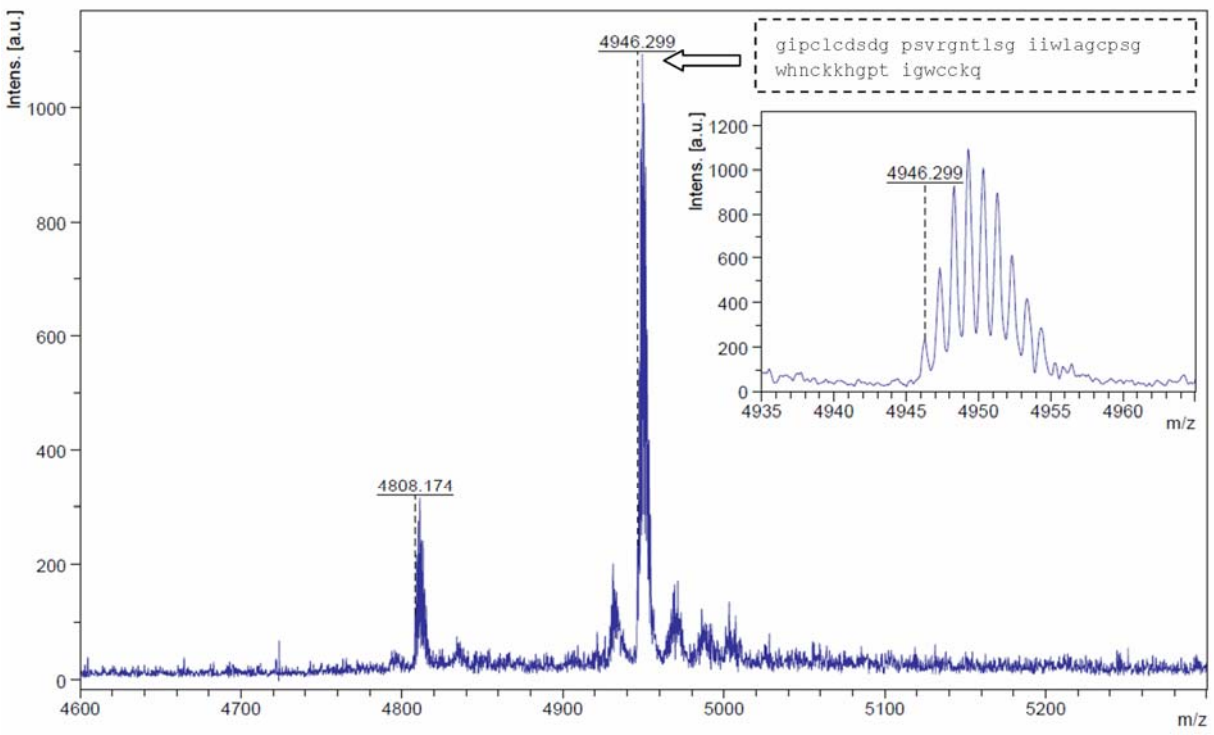

Fig. 5 MALDI-TOF mass spectrum of fraction 5 using the matrix 2,5-DHB in reflectron mode. The peak obtained microsequence of ATX II is showed in the box.

In silico analysis

The *in silico* analysis showed in table 1 allowed to determine the antimicrobial potential of ATX II.

The predicting database used were The Antimicrobial Peptide Database (APD), Collection of Antimicrobial Peptides (CAMP) and Antimicrobial Sequence Scanning System (AMPA) (Wang and Wang, 2004; Shaini *et al*., 2009; Torrent *et al*., 2009, 2012).

APD is based on two characteristic indices for the determination of antimicrobial power: Wimley - White hydrophobicities and Boman Index (Wang and Wang, 2004).

The hydrophobicity index is positive for ATX II and negative for BDS II, indicating that for the first molecule of the amino acids located in that region of the protein are more hydrophobic and therefore there is a greater potential that can play an antimicrobial function.

Even the Boman Index attaches a greater potential antimicrobial ATX II, in fact a lower value of this index (≤ 1) indicates that the peptide has a higher antibacterial activity.

Furthermore, aligning our sequences with those present in this database, ATX II showed a similarity of 35 % with active peptides towards Gram + , Gram- and fungi , while BDS II showed a similarity of 35 % towards Gram + and Gram -.

CAMP uses a algorithm prediction, Support Vector Machine (SVM), incorporated into the database, which also gives a probability score (0 - 1) for the prediction. The higher the probability, the greater the possibility that the prediction may be correct (Shaini *et al.,* 2009). According to this database both molecules, ATX II and BDS II, have the potential to be antimicrobial peptides, with a higher probability for ATX II.

AMPA uses of graphs showing the antimicrobial profile for the sequence of entry (s) and computes the probability of finding stretches of sequence in non-antimicrobial proteins (Torrent *et al.,* 2009, 2012). For BDS II is provided a probability value of 1 % to find the section provided in a non-antimicrobial protein.

Following the analysis *in silico* we can conclude that both molecules have a high probability of being factors and antimicrobials, this probability, it appears to be greater for ATX II.

Subsequently, all the toxins of *A. sulcata* were analyzed by prediction database, showing that neurotoxins specific for sodium channels have a higher chance of being antimicrobial factors compared to neurotoxins specific for potassium channels and, confirming that ATX-II presents all the characteristics suitable to be a factor antimicrobial together with ATX-V, another neurotoxin specific for the sodium channels (Table 1).

Using SWISS-MODEL was verified the possibility of an alignment (ClustalW2) for the homology of structure between ATX II (target) and the chain A of the Human Beta-defensin 2 (1fd3A) (Template). The quality of the model was verified by two programs: Anolea and Qmean.

The alignment of the two sequences was also analyzed with the program DSSP which defines the secondary structure, the geometric characteristics and

Table 1 Summary of the antimicrobial properties of all the known *Anemonia sulcata* neurotoxins

Toxin	Uniprot/Genbank Accession number	Toxin family	Target	Wimley-White hydrophobicities (Kcal/mol) *APD*	Total hydrophobic ratio(%) *APD*	Net charge *APD*	Boman Index (Kcal/mol) *APD*	AMP probability *CAMP*	No-AMP stretch (%) *AMPA*
ATX-I	P01533/ -	Type I	Na_V1	2.54	39	+2	1.15	0.988	0
ATX-II	P01528/ -	Type I	Binds to site 3. $DmNa_V$, SCN2A and SCN5A	1.39	38	+2	0.72	0.983	0
ATX-III	P01535/ -	Sea anemone short toxin family	Na_V1	-0.02	33	+1	0.94	0.984	18
ATX-V	P01529/ -	Type I	Na_V	1.54	39	+3	0.76	0.979	0
SA5 II	P10280/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	-	14.87	32	+4	2.28	0.889	10
kalicludin-1	Q9TWG0/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	$K_V1.2$	13.97	32	+4	2.66	0.579	14
kalicludin-2	Q9TWF9/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	$K_V1.2$	14.51	32	+8	3.01	0.827	5
kalicludin-3	Q9TWF8/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	$K_V1.2$	13.47	33	+4	2.49	0.860	3
BDS-I	P11494/ -	Cnidaria kunitz-type proteinase inhibitor/Type II	$K_V3.1$, 3.2, 3.4	-2.73	34	+3	0.81	0.969	1
kaliseptin	Q9TWG1/ -	Type I	$K_V1.2$	10.76	38	+5	2.08	0.918	16
BDS-II	P59084/ -	Type III	$K_V3.1$, 3.2, 3.4	-1.19	34	+2	0.95	0.928	1

The parameters indicated in red are congruent with neurotoxin antimicrobial properties.

orientation of the protein, showing the presence of alpha helices (h) and folds (s) common to the two molecules.

Although the structural model resulting show of the regions of incompatibility between the two molecules there are large regions compatible with a common structure in terms of energy (Anolea and Qmean) both as a possible presence of secondary structures common.

Discussion

Cnidarian are a source of neurotoxins acting on sodium and potassium ion channels (Yamaguchi *et al*., 2010) or cytotoxic pore forming molecules (Maisano *et al*., 2013; Parisi *et al.*, 2014).

The sea anemone *A. sulcata*, is exposed to attack from predators with the tentacle always exposed. Moreover, *A*. *sulcata* captures food actively wide spectrum of prey (Chintiroglou and Koukouras, 1992), that causes frequently the rupture of the soft tentacle with consequent bacterial infection. Thus, the chemical arsenal of *A*. *sulcata* represents the preferred strategy to survive in such a habitat mainly with the produced neurotoxin together with antimicrobial molecules (Moran *et al*., 2008).

The present study describes isolation and characterization of a neurotoxins that possess also antibacterial activity against *M. lysodeikticus.* This neurotoxin was characterized and purified from the tentacle of an Anthozoa *A. sulcata* by acid extraction, HPLC purifications mass spectroscopy and antibacterial assays.

More than fifty toxins specific for sodium channels classified on the basis of amino acid sequence (Homma and Shiomi, 2006). The types 1 and 2 are composed of a number of amino acid residues between 46 and 49, the type 3 is instead characterized by polypeptide chains ranging from a minimum of 27 to a maximum of 32 amino acid residues (Homma and Shiomi, 2005). A common origin of animal AMPs and toxins seems to be biologically expedient. Despite the difference of their affected targets-the membranes of micro - or macroorganisms, functioning conditions of both peptide groups impose similar requirements to molecule structure and its physicochemical characteristics, such as cationicity, often coupled with amphipathicity, resistance to enzymatic hydrolysis, and overall compactness.

The best-known examples of these are the pardaxins of fish that were originally purified on the basis of their toxic, anti-predatory activity (Lazarovici *et al.,* 1986) yet have similar antibacterial potencies to the amphibian magainins and insect cecropins (Oren and Shai, 1996).

The way these molecules are engaged in defense and aggression mechanisms includes temporary accumulation in reservoirs of specialized cells followed by eventual release to extracellular environment. It would be logical to assume that similar mechanisms of processing and secretion may be involved in both cases requiring the presence of the same signal sequences within peptide precursors. Overlapping of biological properties, along with the sequence homology, might be a consequence of divergent evolution from a common ancestor (Ovchinnikova *et al.,* 2006).

The neurotoxin ATX II is a type 1 sodium channel toxin and consists of 47 amino acid residues, linked by three disulfide bridges. ATX II specifically binds to the sodium channel, thus delaying its inactivation during signal transduction. This molecule has a strong effect on crustaceans and insects and a weaker effect on mammals. The research about these type of toxins specific for sodium channels have been thoroughly investigated also because they constitute a major fraction of the venom (Moran *et al.,* 2009).

Nature thus seems to 'mix and match' useful domains to create AMPs (Smith *et al*., 2010) that could have mechanisms to destroy their targets compatible with other biological function. Cnidarians have impressive strategies for locomotion, feeding and reproduction. Its detailed study may allow better understand the diversification of the molecular novelties of these unique metazoan species (Frazao *et al*., 2012). In the light of the obtained results, we can consider ATX II as a neurotoxin having antimicrobial peptide and functional characteristics. This multi-functionality can be an optimal strategy for survival by allowing these animals to be active predators through the production of neurotoxins and to resist bacterial infections caused eventual rupture of the tentacles through the functionality by antimicrobial peptides.

Acknowledgements

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