Purification and primary structure of two isoforms of arenicin, a novel antimicrobial peptide from marine polychaeta *Arenicola marina*

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Abstract Two novel 21-residue antimicrobial peptides, arenicin-1 and arenicin-2, exhibiting activity against Gram-positive and Gram-negative bacteria and fungi, were purified from coelomocytes of marine polychaeta *Arenicola marina* (lugworm) by preparative gel electrophoresis and RP-HPLC. Molecular masses (2758.3 and 2772.3 Da) and complete amino acid sequences (RWCVYAYVRVRGVLVRYRRCW and RWCVYAYVRIRGVLVRYRRCW)[1] were determined for each isoform. Each arenicin has one disulfide bond (Cys3-Cys20). The total RNA was isolated from the lugworm coelomocytes, RT-PCR and cloning were performed, and cDNA was sequenced. A 202-residue preproarenicin contains a putative signal peptide (25 amino acids) and a long prodomain. Arenicins have no structure similarity to any previously identified antimicrobial peptides.

Keywords: Antimicrobial peptide; Arenicin; Lugworm; Marine invertebrate; Innate immunity; Primary structure; cDNA; Precursor; *Arenicola marina*

1. Introduction

Aquatic invertebrate animals have no acquired immunity with a system of antibodies diversification. They are presumed to use their innate immune system as principal defense against potential pathogens. Endogenous antimicrobial peptides (AMPs) are the earliest molecular factors in the evolution of innate immunity and are considered to play a key role in invertebrate host defense [1,2]. AMPs display broad-spectrum activity against pathogenic bacteria, fungi, and enveloped viruses. Many aquatic species, which live in a microbe laden environment, possess an AMPs-based defense system. A variety of AMPs were discovered and characterized from representatives of aquatic invertebrates, including marine sponge, molluscs, chelicerata, crustaceans, and ascidians. Among them are AMPs from the marine sponge *Discodermia kiiensis* [3]; from hemocytes and plasma of the mussels *Mytilus galloprovincialis* and *Mytilus edulis* [4–6], from skin and mucus of the sea hare *Dolabella auricularia* [7]; from hemocytes of the Japanese horseshoe crabs *Tachypleus tridentatus* [8] and *Limulus polyphemus* [9], of the shore crab *Carcinus maenas* [10,11], of the blue crab *Callinectes sapidus* [12], and of the shrimp *Litopenaeus* (*Penaeus*) *vannamei* [13]; from the solitary tunicates *Styela clava* [14–16], *Styela plicata* [17], and *Halocynthia aurantium* [18,19]. The described AMPs include the cysteine-rich peptides of mussels (myticin) [5] and horseshoe crabs (tachyplesins and polyphemusins) [8], the proline and cystein–rich peptides from shrimp (penaeidins) [13], the α-helical peptides from ascidians (clavanins, styelins) [14–16]. Antimicrobial peptides from marine organisms could afford design of new antibiotics manifesting broad-spectrum antibacterial activity in the presence of physiological or elevated NaCl concentrations.

We have discovered a new family of small (21-residue) antimicrobial peptides, termed arenicins, in coelomocytes of marine polychaeta lugworm *Arenicola marina*. These AMPs exhibited activity against Gram-positive, Gram-negative bacteria and fungi. Arenicins have a single disulfide bond Cys3–Cys20, forming a 18-residue ring, and have no sequence homology to any previously identified AMPs. The purification, primary structure, antibiotic properties and structure of the preproarenicins mRNA are described in this report.

2. Materials and methods

2.1. Materials

All solutions were prepared using water from a Milli-Q plus 185 apparatus (Millipore). Solvents were of high performance liquid chromatography (HPLC) grade. All reagents were of analytical grade.

2.2. Preparation of coelomocytes of the marine polychaeta Arenicola marina

Lugworms *A. marina* were collected at a coastal zone (the island Sredniy, White Sea, Russia) in the middle of August when reproduction season of polychaeta was over. Coelomic fluid was harvested by puncturing the worm body wall with a sterile syringe and kept at 4 °C. Plasma was separated by centrifugation at $400 \times g$ for 10 min. Coelomocyte pellets were stored in a freezer.

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[1] The protein sequence data reported in this paper will appear in the Swiss-Prot and TrEMBL knowledgebase under the accession numbers P84105 for arenicin-1 and P84106 for arenicin-2.

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2.3. Purification of antimicrobial peptides from coelomocytes

Before purification procedure the coelomocytes were thawed, resuspended in 10% acetic acid (1 g per 10 ml of 10% CH_3COOH), homogenized and stirred overnight at 4 °C. Centrifugation (25 000 × *g*, 2 h, 4 °C) was used to clarify the supernatant which was taken for further purification procedure. Supernatant was passed under pressure through a stirred ultrafiltration cell (Amicon, Danvers MA) equipped with a YM-10 filter. The ultrafiltrate was dried in vacuo (Speed Vac Concentratror CS-18, Savant Instruments, Hickville, NY) and applied to preparative continuous acid–urea PAG electrophoresis [20]. Aliquots of each fraction were tested for antimicrobial activity by radial agar-diffusion assay. Active fractions were purified by reverse-phase HPLC on a Vydac C-18 column with a linear gradient of acetonitrile concentration from 0% to 60% (1% min^{-1}) in 0.1% TFA. The peptides of interest were examined by gel electrophoresis.

2.4. Gel electrophoresis

Tricine–SDS PAGE [21] and acid–urea PAGE [22] were performed in minigels using a SE 250 vertical gel unit (Hoefer, San Francisco, CA). Gels were stained with a solution that contained 1 g of Coomassie brilliant blue R-250 (Sigma), 270 ml methanol, 630 ml water and 150 ml formaldehyde, and were then destained in 5% acetic acid.

2.5. Antimicrobial assay

Antimicrobial activities of the peptides were measured in radial diffusion assays by the gel overlay techniques [23]. Samples were tested for antimicrobial activity against *Escherichia coli* strain ML-35p, *Listeria monocytogenes* strain EGD and *Candida albicans* strain 820. The underlay agars used for all organisms had a final pH of 6.5 and contained 9 mM sodium phosphate, 1 mM sodium citrate, 1% w/v agarose and 0.3 mg/ml of trypticase soy broth powder (BBL, Cockeysville, MD). 10 units of activity in radial diffusion assay correspond to a 1 mm diameter clear zone around the 3 mm sample well. Purified arenicin-1 and arenicin-2, and porcine protegrin-1 were dissolved in 0.01% acetic acid at a concentration of 120 μg/ml, and six additional 2-fold serial dilutions in 0.01% acetic acid were performed. 5 μl of each solution was tested in thin agarose gels. MIC were defined in low (10 mM sodium phosphate buffer, pH 7.4) and high (10 mM sodium phosphate buffer, 100 mM NaCl) concentrations of salt.

2.6. Molecular mass determination

The molecular masses of the purified AMPs were determined by matrix-assisted laser desorption ionization mass spectromentry (MALDI-MS) with a Vision 2000 mass spectrometer (ThermoBio-

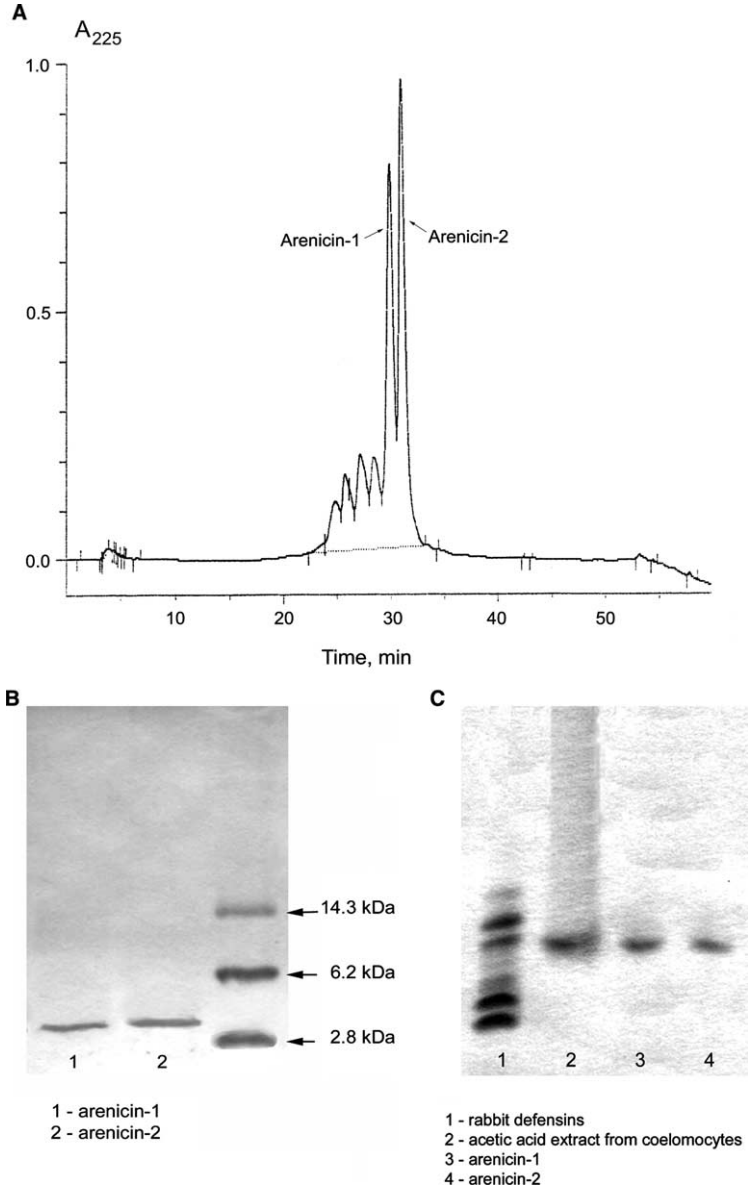

Fig. 1. (A) HPLC purification of arenicins; (B) tricine–SDS PAGE; and (C) acid–urea PAGE.

Analysis, UK). 0.15 M of 2,5-dihydroxybenzoic acid was used as a matrix in 25% methanol and 0.1% TFA.

2.7. Peptide microsequencing

The N-terminal amino acid sequences were determined using the Procise cLC 491 Protein Sequencing System (PE Applied Biosystems, USA). Phenylthiohydantoin derivatives of the amino acids were identified by the 120A PTH Analyzer (PE Applied Biosystems, USA).

2.8. Reduction and alkylation reaction

Peptide samples were denaturated in 6M guanidinium chloride, 0.2 M Tris–HCl, pH 8.5, and reduced by incubation with a 10:1 molar excess of dithiothreitol at 25 °C for 4 h under N_2 atmosphere, and alkylated using a 5:1 molar excess of 4-vinylpyridine at 25 °C for 3 h under N_2 atmosphere. The *S*-pyridylethylated peptides were desalted by reverse-phase HPLC on a Vydac C-4 (4.6 × 250 mm, 5 μm) column with a linear gradient of acetonitrile concentration from 0% to 60% ($1\%\,min^{-1}$) in 0.1% TFA at a flow rate of 0.5 ml/min.

2.9. Total RNA isolation

To preserve RNA before total RNA isolation, the dissected fresh tissues of the lugworm *A. marina* were submerged in RNA stabilization solution RNAlater™ (Ambion Inc., Austin, TX, USA), which rapidly penetrates tissues, inactivates nucleases and protects RNA from degradation prior to nucleic acid isolation. Samples were placed in 10 volumes of RNAlater and stored at −20 °C. Frozen samples were thawed, removed from the solution and homogenized in liquid nitrogen. Intact total RNA was isolated by using SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA).

2.10. Reverse transcription-PCR

To determine the structure of the 3′ and 5′ ends of arenicin cDNA, the RACE strategy was used. RT-PCR experiments were conducted with SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA, USA). Two degenerate gene-specific primers for 3′RACE were designed corresponding to the amino acid sequence of arenicins -Cys^3-Val^4-Tyr^5-Ala^6-Tyr^7-Val^8-Arg^9- determined by N-terminal automated amino acid sequencing of the pyridylethylated peptides:

No. 1 5′-TGC GTC TAC GC(T,C) TA(T,C) GT(T,C) (A,C)G-3′

No. 2 5′-TGC GTC TAC GC(T,C) TA(T,C) GT(A,G) (A,C)G-3′.

The following thermal cycle profile was used for 3′RACE PCR: 95 °C for 90 s, 35 cycles of 94 °C for 30 s, annealing at 50 °C for 40 s, and elongation at 72 °C for 120 s. Amplification of 5′ cDNA end was performed with two gene-specific primers complementary to the 3′ untranslated region:

No. 3 5′-TCT GAG GGC TGC AGA ATC GTC GGT-3′

No. 4 5′-GTA GAA AAT GTC ATT GAT CCC ATT TCA-3′.

In this case, the thermal cycle profile was slightly modified by increasing the annealing temperature up to 64 °C. Products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide and viewed with a UV transilluminator.

2.11. RT-PCR products cloning and sequencing

The main PCR products were eluted from low melting point agarose gel and cloned in pGEM-T vector (Promega). Basic recombinant DNA techniques were exploited as described [24]. *E. coli* strain DH-10B (Life Technologies, Gaithersburg, MD) competent cells were transformed and a miniscale preparation of plasmid DNA was carried out using the alkaline lysis method. Nucleotide sequencing analysis was performed by dideoxy chain termination technique in double stranded vector using the fluorescence-labeled dye terminator method and an ABI PRISM 3100-Avant automatic sequencer (Applied Biosystems, USA).

3. Results

3.1. Purification of AMPs from coelomocytes of Arenicola marina

To identify and characterize the AMPs from marine polychaeta *A. marina*, they were isolated from the coelomocytes of the lugworm in acidic conditions. The coelomocytes were separated by centrifugation, kept in a freezer, then thawed, resuspended in 10% acetic acid and homogenized. After centrifugation, the obtained supernatant was taken for the further purification procedure by ultrafiltration via Amicon YM-10 filter. The ultrafiltrate was dried in vacuo and applied to

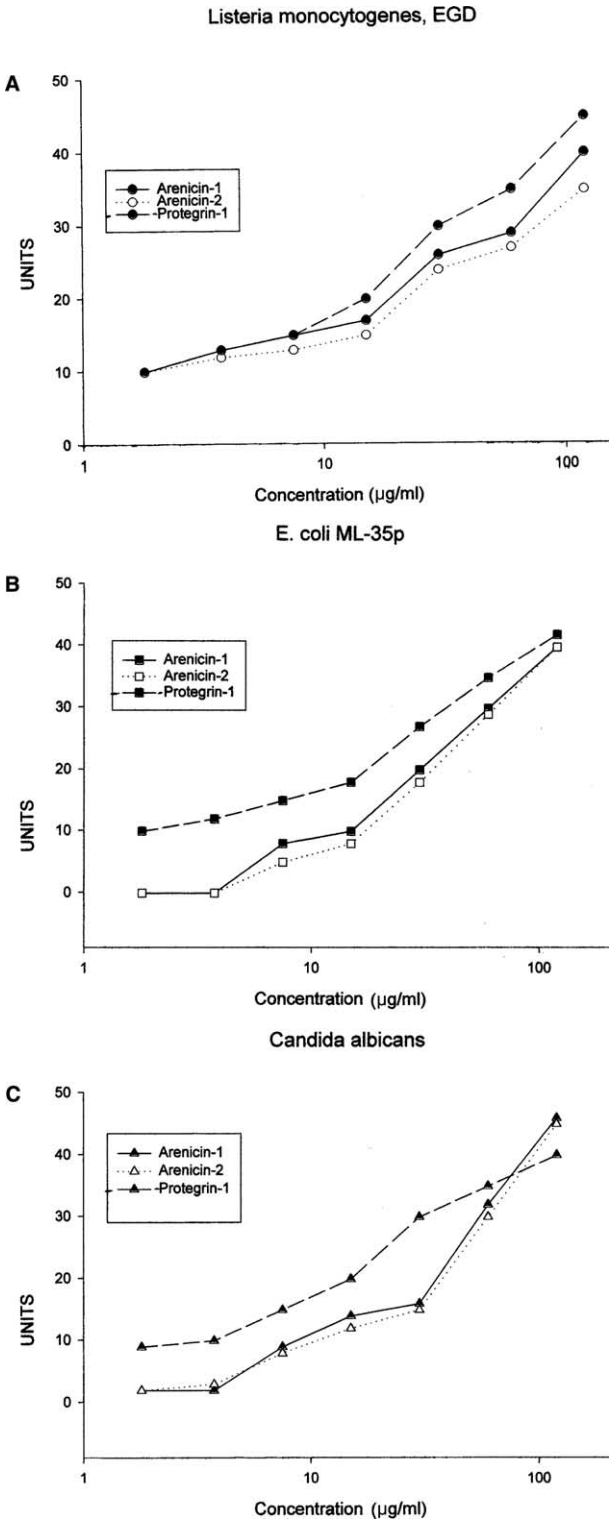

Fig. 2. Antimicrobial activity of arenicins (A) against *L. monocytogenes*; (B) against *E. coli*; and (C) against *C. albicans.*

preparative continuous acid–urea PAG electrophoresis. Aliquots of each fraction were tested for antimicrobial activity by radial agar-diffusion assay. Active fractions were purified by reverse-phase HPLC on a Vydac C-18 column with a linear gradient of acetonitrile concentration (Fig. 1). The peptides of interest were examined by gel electrophoresis in tricine–SDS PAGE (Fig. 1A) and acid–urea PAGE (Fig. 1B). RP-HPLC procedure resulted in purification to homogenety of two individual lugworm peptides. The peptides were named arenicin-1 and arenicin-2 according to their elution order from the HPLC column.

3.2. Biological activity of arenicins

Both arenicins were shown to be active against Gram-positive *L. monocytogenes*, strain EGD (Fig. 2A), Gram-negative *E. coli*, strain ML-35p (Fig. 2B) and fungi *C. albicans*, strain 820 (Fig. 2C). Antimicrobial activities of both arenicins were absolutely equal in all tests. At doses exceeding 25 μg/ml, both arenicins inhibited growth of the microorganisms with effectiveness comparable with that of one of the most strong peptide antibiotics protegrin-1 [25]. Under low salt conditions, arenicins and protegrin-1 had approximately equal activities against *L. monocytogenes* (MIC 0.6 and 0.8 μg/ml, respectively), but arenicins were less potent against *E. coli* (MIC 4 μg/ml) and *C. albicans* (MIC 4.5 μg/ml) than protegrin-1 (MIC 0.7 and 0.8 μg/ml, respectively). The presence of 100 mM NaCl did not influence on activity of arenicins against *E. coli* and of protegrin-1 against all tested microorganisms. The activity of arenicins against *C. albicans* was strongly reduced (MIC 20 μg/ml). It is interesting to note that the activity of arenicins against *L. monocytogenes* also increased slightly (MIC 0.4 μg/ml).

3.3. Molecular characterization of arenicins

MALDI mass analysis of the purified arenicins revealed two different molecular ion masses at *m/z* 2758.1 and *m/z* 2772.3. Reduction of arenicins with dithiothreitol and alkylation with vinylpyridine led to mass shifts of 212.2 Da for arenicin-1 and of 212.4 Da for arenicin-2. These results demonstrate that the two cysteine residues of each arenicin are engaged in one disulfide bridge. A partial 20 residue N-terminal amino acid sequence of each *S*-pyridylethylated (PE) arenicin was obtained by automatic Edman degradation: Arg^{1}-Trp^{2}-$PECys^{3}$-Val^{4}-Tyr^{5}-Ala^{6}-Tyr^{7}-Val^{8}-Arg^{9}-Val/Ile^{10}-Arg^{11}-Gly^{12}-Val^{13}-Leu^{14}-Val^{15}-Arg^{16}-Tyr^{17}-Arg^{18}-Arg^{19}-$PECys^{20}$-X^{21}.

In order to obtain the complete arenicins sequences, the cDNA sequences were determined.

3.4. Total RNA isolation, RT-PCR, cloning and sequencing

The fresh tissues of the lugworm *A. marina* were submerged in RNA stabilization solution and then homogenized in liquid nitrogen. Total RNA was isolated by using spin columns purification system. To determine the structure of the 3′ and 5′ ends of arenicin cDNA, the RACE strategy was used. A set of 3′RACE reactions using two degenerate primers specific for mature arenicins showed a main band of approximately 600 bp in both cases. Cloning and sequencing of the fragment acquired with primer No. 1 revealed the C-terminal sequence of mature arenicin-1 and the 3′UTR of its cDNA. The remaining part of full-length cDNA for arenicin-1 was determined by the following 5′RACE experiments. At this stage, the 5′-terminal part of cDNA coding for arenicin-2 was also obtained. Both sequences (GenBank Accession Nos.: AY684856, AY684857) included a 609-bp open reading frame encoding a peptide precursor of 202 amino acid residues (Fig. 3). The 5′UTR and

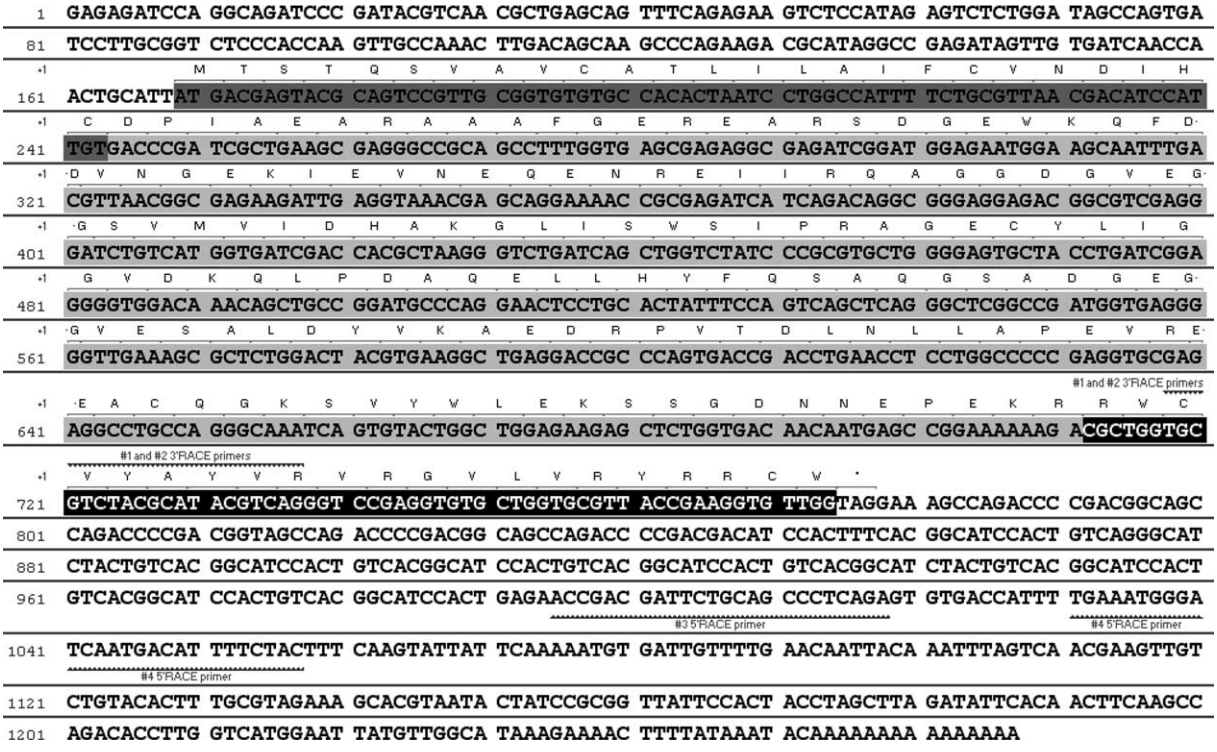

Fig. 3. Sequence of mRNA encoding preproarenicin-1 and its translation. The open reading frame is represented with the following individual components: signal peptide (dark gray), prosequence (light gray); and mature peptide (black).

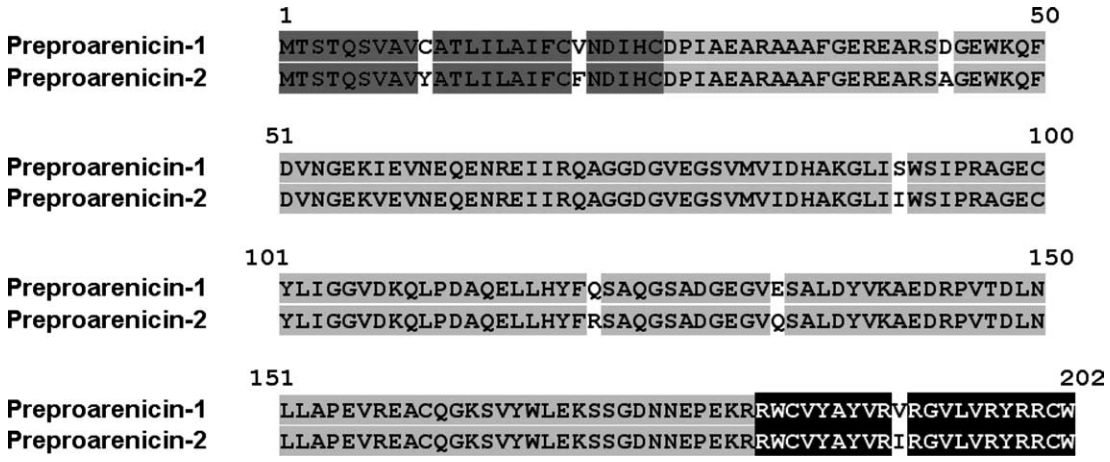

Fig. 4. Differences between arenicin-1 and arenicin-2 precursors. The mature peptides are shown in black, signal peptides in dark gray; and substituted amino acids are highlighted.

translated regions of two isoforms have approximately 98.5% of homology (data not shown). Besides Val/Ile interchange within the mature peptide, 12 other nucleotide substitutions, leading to six additional amino acid changes in a precursor molecule, were detected (Fig. 4). Noteworthy, two clusters of tandem repeats were found within the 3′UTR just after the stop codon. This fact was taken into account when designing primers for 5′RACE. BLAST search of protein and nucleic acid databases (http://www.ncbi.nlm.nih.gov/BLAST) did not reveal significant similarities with known sequences. The sequence analysis using SignalP (http://www.cbs.dtu.dk/services/SignalP/) pointed out the Cys^{25}–Asp^{26} bond as the most probable cleavage site for eukaryotic signal peptidases [26,27].

4. Discussion

The present study describes the isolation and characterization of two novel 21-residue antimicrobial peptides, arenicin-1 and arenicin-2, which exhibited activity against Gram-positive, Gram-negative bacteria and fungi. Arenicins were purified from coelomocytes of marine polychaeta *A. marina*. Complete amino acid sequences (RWCVYAYVRVRGVLVRYRRCW and RWCVYAYVRIRGVLVRYRRCW) were determined for each isoform. The calculated molecular masses of the arenicin-1 (2758.32 Da) and arenicin-2 (2772.35 Da) matched the measured masses of the isolated peptides (2758.1 and 2772.3 Da).

AMPs containing a single disulfide bond earlier were isolated from frog skin (brevenins [28], pipinins [29], esculentins [30], ranalexins [31], ranatuerins [32], gaegurins [33], rugosins [34], palustrins [35], nigrocins [36], and japonicins [37]). All these peptides are characterized by a single intramolecular disulfide bridge between two cysteine residues forming a small 6–8-residue ring at the C-terminal part of the molecule. A 21-residue peptide thanatin, containing a single disulfide bridge, forming an octapeptide ring, was isolated from the hemipteran insect *Podisus maculiventris* [38]. Cationic antimicrobial dodecapeptide with one disulfide bond, forming a nonapeptide ring, was purified from bovine neutrophils [39]. Four broad-spectrum, 11 and 12 residue antimicrobial peptides, isolated from skin secretions of the Indian frog *Rana tigerina* and named tigerinins, are characterized by a single intramolecular disulfide bridge, forming a nonapeptide ring [40]. Database searches did not show sequence similarities of arenicins with any known AMP. Both arenicins have a single disulfide bridge (Cys3–Cys20), forming a large 18-residue ring. In the light of these results, we can consider arenicins as the first example of a new class of antimicrobial peptides.

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