Fish and Shellfish Immunology 88 (2019) 352–358

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Molecular characterization, recombinant expression and bioactivity profile of an antimicrobial peptide, Ss-arasin from the Indian mud crab, *Scylla serrata*

A. Anju[a,*], C.K. Smitha[a], K. Preetha[a], R. Boobal[b], Philip Rosamma[a]

[a] Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, P B. No 682016, Kochi, India
[b] National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Kochi 16, Kerala, India

ARTICLE INFO

Keywords:
Scylla serrata
Ss-arasin
Gene expression
Antibacterial activity
Anticancer activity

ABSTRACT

Antimicrobial peptides (AMP) are potential alternatives to conventional antibiotics with the prospect to treat infections caused by multidrug resistant bacteria. This is the report of the first arasin sequence from the mud crab, *Scylla serrata*, designated as Ss-arasin. The complete cDNA sequences of the open reading frame (ORF) is comprised of 198 bp encoding 65 amino acid with a predicted molecular weight of 7 kDa and a predicted isoelectric point of 10.68. The sequence of the N-terminal 24 amino acid residues is indicative of a signal sequence directing the newly synthesize protein toward the secretory pathway. The 41-residue mature peptide is composed of two domains, an N-terminal Gly/Arg-rich domain and a C-terminal cysteine-rich domain. Challenging the mud crab with lipopolysaccharide (LPS) increased expression of Ss-arasin mRNA in haemocytes, reaching the highest level at 6 h, before dropping to basal levels at 24 h. Recombinant rSs-arasin showed antimicrobial activity against three bacterial species *Staphylococcus aureus* (40 mM), *Pseudomonas aeruginosa* (40 mM) and *Escherichia coli* (40 mM) implying significant anti-bacterial action. In addition, recombinant rSs-arasin inhibited human cervical carcinoma (HeLa) and colon carcinoma (HT-29) cell growth. These initial findings are encouraging to further study the structure-activity relationships to optimize these biological functions for future drug development.

1. Introduction

One of the biggest threats in modern medicine is the emergence of multi-drug resistant bacteria. Almost all the traditional antibiotic classes [1] are being affected. Because the window of choices of treating multidrug resistant bacterial infections is closing, finding alternative drugs has high priority for health professionals around the globe. A promising route was opened with the discovery of antimicrobial peptides (AMPs). In difference to traditional antibiotics, which are predominantly secondary metabolites produced by microorganisms, antimicrobial peptides are gene encoded ribosomally synthesized peptides with multi-functional role especially in the innate defense against microbial invasion. Although their molecular function is still debated the two mechanisms discussed in the literature are attributed to either membrane disruption by pore-formation or by permeating the membrane and acting on intracellular targets.

Structurally diverse AMPs are organized into three major groups: (i) linear peptides that form amphipathic α-helices, (ii) cysteine-rich peptides where all thiol groups are oxidized to form disulphide bonds and (iii) peptides rich in certain amino acids, such as proline, arginine, glycine, for instance Refs. [2,3]. Some AMPs are chimera where the sequence features of two classes are combined. For instance, penaeidins, found in shrimps and prawns, the N-terminal domain belongs to the class of Pro/Arg-rich peptides, while the C-terminal domain belongs to the class of cysteine-rich peptides, containing six Cys residues connected by three disulphide bridges [4–6]. Similarly, arasins, isolated from *Hyas araneus* haemocytes, possess Pro/Arg-rich N-terminal regions and two disulphide connections in the C-terminal regions [7].

Invertebrate organisms that lack adaptive immunity are a rich and mostly untapped source of AMPs. In particular crustaceans are of interest because of their biological diversity and their ecological and commercial importance. Here we describe our results of AMP arasin from the mud crab, *Scylla serrata* (Ss-arasin). In this study we identified and characterized the sequence coding for Ss-arasin isolated from haemocytes. The protein was recombinantly expressed and antimicrobial and anticancer activity confirmed using various bacteria and

* Corresponding author.
E-mail address: anju.moonjely@gmail.com (A. Anju).

https://doi.org/10.1016/j.fsi.2019.03.007
Received 3 November 2018; Received in revised form 2 March 2019; Accepted 3 March 2019
Available online 06 March 2019

cultures of mammalian cancer cell lines.

2. Materials & methods

2.1. Experimental animal and sample collection

Live animal of adult mud crab, *Scylla serrata* was collected from Cochin harbour in Kalamukku, India. The animals were taken alive in tanks with circulating seawater. Animals were acclimatized for a week before they were used. Haemolymph was collected from the base of abdominal appendages using specially designed capillary tubes (RNase-free) rinsed with pre-cooled anticoagulant solution (10% RNase free sodium citrate pH 7.0). Haemocytes were collected by centrifugation at 800 × *g* at 4 °C for10 min and stored at −80 °C until use.

2.2. RNA isolation and cDNA cloning

Total RNA was extracted from the haemocytes using NucleoSpin RNA II reagent (Macherey-NagelGmbH & Co, Germany) as per the manufacturer's instructions and stored at −80 °C until further use. The RNA concentration was determined spectrophotometrically at 260 and 280 nm cDNA was synthesized with High Capacity RNA-to-cDNA kit (Takkara, Japan) in accordance with the manufacturer's protocols. First strand cDNA was generated in a 20 µl reaction volume containing 2 µg of total RNA, 10 µl (2×) RT buffer, 1 µl (20×) enzyme mix. The reaction mix was incubated at 37 °C for 60 min followed by an inactivation step at 85 °C for 5 min. PCR amplification was carried out using forward and reverse primers (Table 1) designed using Beacon designer (Bio-rad) software based on the sequence information of Mud Crab, *Scylla paramamosain* available in the GenBank data (HM345951). Polymerase chain reactions (PCR) were carried out to obtain the open reading frame (ORF) and untranslated region (UTR) of *S. serrata*. The reaction volume of 20 µl consisted of 10 µl of Emerald master mix (TaKaRa, Japan), 1 µl of each primer (10 mM), 7 µl of PCR-grade water and 1 µl of cDNA. The PCR program consisted of an initial denaturation of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30s, 55 °C for 30s, 72 °C for 45s and the final extension step of 72 °C for 7 min. PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide (0.5 µg/ml). The PCR product was eluted, cloned into the pGEM®-T Easy cloning vector (Promega) and transformed into competent *E. coli* DH5α cells. Positive clones were identified as white colonies on LB (Luria broth) agar and were used for sequencing in both directions.

2.3. Sequence analysis

The sequence was analyzed for identity and similarity to known sequences by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and multiple sequence alignment was generated using the CLUSTALW program (http://www.ebi.ac.uk/clustalw/index.html). Gene translation and prediction of deduced proteins were performed with ExPASy (http://www.au.expasy.org/). PROTPARAM software was used to predict the molecular weight and isoelectric point of the peptides. Signal peptide prediction was performed by SignalP software version 3.0 (http://www.cbs.dtu.dk/services/SignalP/). PROSITE Database was used to identify the Domains and motifs in peptide sequence (http://prosite.expasy.org/scanprosite/).

Table 1
PCR primers used in the present study.

Gene	Sequence(5′- 3′)
Ss-arasin-F	ACTTCGACATGGAGCGACGCAC
Ss-arasin-R	TTAAGTCAAAGAAGCAAGAGTCAAC
pET- 32a(+) arasin-F	TAAGCAGGATCCATGTCGCCTCGGGTGAGACGC
pET- 32a(+) arasin-R	TAAGCAAAGCTTTTATTAGCCGTAATTGGCACAAGG
Ss-arasin-real time-F	CTGCTGATTGTCCTGCTA
Ss-arasin-real time-R	ACACGGTGACCTGATACA
β-actin-F	GCCCTTCCTCACGCTATCCT
β-actin-R	GCGGCAGTGGTCATCTCCT

2.4. Immune challenge

Fifteen individual animals were injected with a dose of 0.5 mg/kg of LPS (*E. coli* 055:B5, #L2880, Sigma, USA) dissolved in PBS at a concentration of 10 µg/ml. For control 15 individuals were injected with an equal volume of PBS. Experimental and control animals were divided into 5 groups, respectively and reared in individual tanks under identical conditions. At time points (0, 3, 6, 12 and 24 h), haemolymph from three individuals in both groups were withdrawn from the third pereiopod using a syringe and centrifuged at 800 × *g* at 4 °C for10 min to harvest the haemocytes. Haemolymph sampling and collection of haemocytes from three individual samples was performed according to a method previously reported [8]. The haemocyte pellets were immediately used for RNA extraction.

2.5. Real-time PCR

Total RNA was extracted from haemocytes using NucleoSpin RNA II reagent (Macherey-Nagel GmbH & Co, Germany) and the extracts stored at −80 °C until further use. cDNA was synthesized from RNA with primeScript™RT reagent kit (TaKaRa, Japan). The SYBR Green based real-time PCR assay was carried out in a StepOnePlus Real Time PCR system (Applied Biosystem). The amplifications were performed in 96-well plates with 25 µl reaction volume containing 12.5 µl of 2× Power SYBR premix Ex Taqll (2×) Master Mix (TaKaRa, Japan), 0.5 µl (each) of the forward and reverse primers (5 µM), 1 µl of template and 10.5 µl of water. The β-actin gene was used as endogenous control. Real time primer pair for amplification of the Ss-arasin transcript was designed by the Beacon designer (Bio-rad) software shown in Table 1. The thermal profile for the SYBR Green real-time PCR was 95 °C for 10 min followed by 35 cycles of 95 °C for 15 s and 60 °C for 1 min. In a 96-well plate, each sample was run in triplicate. DEPC-water replacing the template was used as the negative control. The absence of non-specific products was confirmed by dissociation curve analysis between 60 and 95 °C.

2.6. Statistical analysis

Multiple comparisons using Duncan's test were made to check the differences between the gene expression in the control and challenged sample using SPSS13.0 software.

2.7. Prokaryotic expression and purification of Ss-arasin protein

The cDNA fragment of Ss-arasin was amplified using primers pET-32a(+) arasin-FP & pET-32a(+) arasin-RP (containing restriction recognition sites for BamHI and HindIII, respectively, Table 1). The reaction volume of 20 µl composed of 10 µl of Emerald master mix (Takkara, Japan), 1 µl of each primer (10 mM), 7 µl of PCR-grade water and 1 µl of cDNA. The PCR program consisted of an initial denaturation of 94 °C for 5 min, followed by 30 cycles of 94 °C for 30s, 55 °C for 30s, 72 °C for 45s and the final extension step of 72 °C for 7 min. PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. The PCR product was eluted, cloned into the pGEM®-T Easy cloning vector and transformed into competent *E. coli* DH5α cells. Positive clones were identified as white colonies on LB (Luria broth) agar, the plasmid isolated and the insert sequenced in both directions. The insert was removed and subcloned into the BamHI/HindIII site of pET-32a (+) vector (Novagen, UK). The sequence of the recombinant plasmid was verified and the plasmid transformed into Rosettagami™B (DE3) pLysS (Novagen, UK). Rossettagami™bacteria carrying pET-32a

(+) Ss-arasin were cultivated in LB broth (50 ml) containing 50μg/ml ampicillin and kanamycin (15 μg/ml) at 37 °C. When the culture reached OD600 = 0.6, Ss-arasin expression was induced by 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 3 h at 37 °C, the cells were collected via centrifugation at 8000 rpm, 4 °C for 10 min. Cells were treated 8 M urea according to standard protocols (Ni-NTA Spin Columns, Qiagen). Solubilized protein under denaturing condition was bound to Ni-NTA-column. The purified protein was eluted with 6 M urea in 50 mM Tris/HCl, pH 7.4, containing 50 mM NaCl, 10% glycerol, 1% glycine, 1 mM EDTA, 0.2 mM oxidized glutathione and 2 mM reduced glutathione. The eluted protein was concentrated to one tenth of its original volume using Millipore's Amicon Ultra-Centrifugal 3 kDa cut-off membrane by centrifuging at 5000 × *g* for 30 min. The concentrated protein was reconstituted to the original volume using the refolding buffer 50 mM Tris-Cl pH 7.4. The buffer exchange process was repeated 10 times to remove the denaturing reagents and thus refold the protein to its native form (yield 2 mg protein/l culture). The pET-32a(+) vector containing thioredoxin tag without insert was selected as negative control. The concentration of trx-His_6-recombinant rSs-arasin fusion protein (in the following named rSs-arasin) and recombinant rTrx protein was quantified by Quant-iT™ protein assay kit using Qubit fluorometer (Invitrogen, UK).

2.8. Antimicrobial assay

The antimicrobial activity of rSs-arasin peptide was examined by a liquid growth inhibition assay [9–11]. Microorganisms used for the antimicrobial activity assay were *Staphylococcus aureus* (MTCC 3061), *Pseudomonas aeruginosa* (MCCB 119) and *Escherichia coli* (MTCC 483). The activity of peptide was determined by the minimal inhibitory concentration (MIC) method. Serial two-fold dilutions of the peptide from 0 to 40 μM were made in 50 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) buffer pH 7.4. Ten microliters of each peptide concentration was added to each corresponding well of a 96-well microtiter plate, each well was inoculated with 10 μl of a bacterial suspension (10^4 CFU/ml) and incubated for 2 h. Thereafter 80 μl of LB media was added to each well. The negative control was performed using recombinant thioredoxin (see above). Cultures were grown for 24 h at 37 °C and the growth of bacteria in each culture were evaluated by computing the absorbance at 600 nm using a Microplate reader (Table 2).

2.9. In vitro cytotoxicity assay

Human lung cancer cell line HT-29 (HTB-38) and cervical cancer cell line HeLa (CCL-2) were procured and cultured in Dulbecco's modified Eagle's medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (Sigma-Aldrich), L-glutamine, sodium bicarbonate and 100 IU/ml penicillin, 100 μg/ml streptomycin and amphotericin B (2.5μg/ml) in a water-saturated atmosphere containing 5% CO_2 at 37 °C. Cells were detached with 1 ml trypsin/EDTA (Invitrogen) for 1 min at 37 °C, 8 ml of complete medium was added and the cells collected by low-speed centrifugation. The cell pellet was suspended in culture medium and 10^7 cells were used to seed a 75-cm^2 culture flask.

Table 3
Microtiter plate read of MTT assay and the corresponding percentage of inhibition in HT-29 and HeLa cell lines.

Sample Concentration (μM)	Average OD at 540 nm (HT-29)	% of inhibition of HT-29 cell lines	Average OD at 540 nm (HeLa)	% of inhibition of HeLa cell lines
3.816	0.4704	57.1	0.4774	50.67
1.908	0.6481	40.89	0.5229	45.96
0.954	0.7172	34.59	0.5444	43.74
0.477	0.7776	29.08	0.6336	34.53
0.238	0.8425	23.16	0.6812	29.61
Control	1.0964		0.9677	

One hundred microlitre of a cell suspension (5×10^4 cells/ml) were added into each well of a 96 well tissue culture plate. After incubation for 24 h at 37 °C, the cells were washed twice with phosphate buffered saline (PBS) and the medium was replaced with fresh DMEM without fetal bovine serum but containing peptide at concentrations of 0.238 μM, 0.477 μM, 0.954 μM, 1.908 μM, and 3.816 μM (Table 3).

Cells were incubated for 24 h at 37 °C and thereafter evaluated for morphological changes under an Inverted phase contrast microscope (Leica, Germany) (Fig. 6). Fifteen milligram of 2, 3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (MTT Sigma, M-5655) was completely dissolved in 3 ml PBS and sterile filtered. The culture medium was replaced and 30 μl of the MTT solution was added. After incubation for 4 h at 37 °C in water saturated air/5% CO_2 atmosphere the absorbance at 540 nm was recorded using a microplate reader [12]. Experiments were conducted in duplicate. The results are expressed as a percentage of the inhibition rate for viable cells. The IC_{50} was calculated at the peptide concentration that induced 50% growth inhibition with respect to the untreated control.

3. Results and discussion

3.1. Molecular cloning and characterization of Ss-arasin

A cDNA sequence with homology to the arasin of crustaceans was identified in the haemocytes of the mud crab *S. serrata* (Fig. 1b). The 305 bp nucleotide sequence consisted of 8 bp at 5′partial UTR and 100 bp at 3′UTR sequences (Fig. 1a). The ORF of cDNA encoded a polypeptide of 65 amino acid with a predicted N-terminal signal peptide of 24 amino acid followed by 41 amino acid of the mature peptide (GenBankMK044533). This indicated that Ss-arasin is a secreted protein with an Arg- and Gly-rich 27-residue N-terminal region and a 14-residue cysteine-rich C-terminal region where the 4 cysteines form two disulphide connections. The molecular weight of the peptide is 7 kDa and a predicted isoelectric point is 10.68. The amino acid sequences of Ss-arasin and arasin-like*Sp* from *S. paramamosain* [7] is 98% identical but differs from the sequences of arasin-2 (54%) from *Hyas araneus*. The cysteine-pattern CXCXXXPCPC of the C-terminal domains is conserved in all known arasin-like species and the disulfide links are assumed to be as determined for arasin [11].

Comparing the amino acid frequencies of the N-terminal domains

Table 2
Microtiter plate read of liquid growth inhibition assay and the corresponding percentage of inhibition in different organisms.

Sample Concentration (μM)	Average OD at 600 nm of *S. aureus*	% of inhibition of *S. aureus*	Average OD at 600 nm of *P. aeruginosa*	% of inhibition of *P. aeruginosa*	Average OD at 600 nm of *E. coli*	% of inhibition of *E. coli*
40	0.148	59.4	0.191	51	0.15	58
20	0.179	50.9	0.21	46	0.183	49
10	0.186	49	0.273	30	0.205	43
5	0.256	29.8	0.312	20	0.252	30
2.5	0.276	24.3	0.315	19.2	0.291	19.4
control	0.365		0.39		0.36	

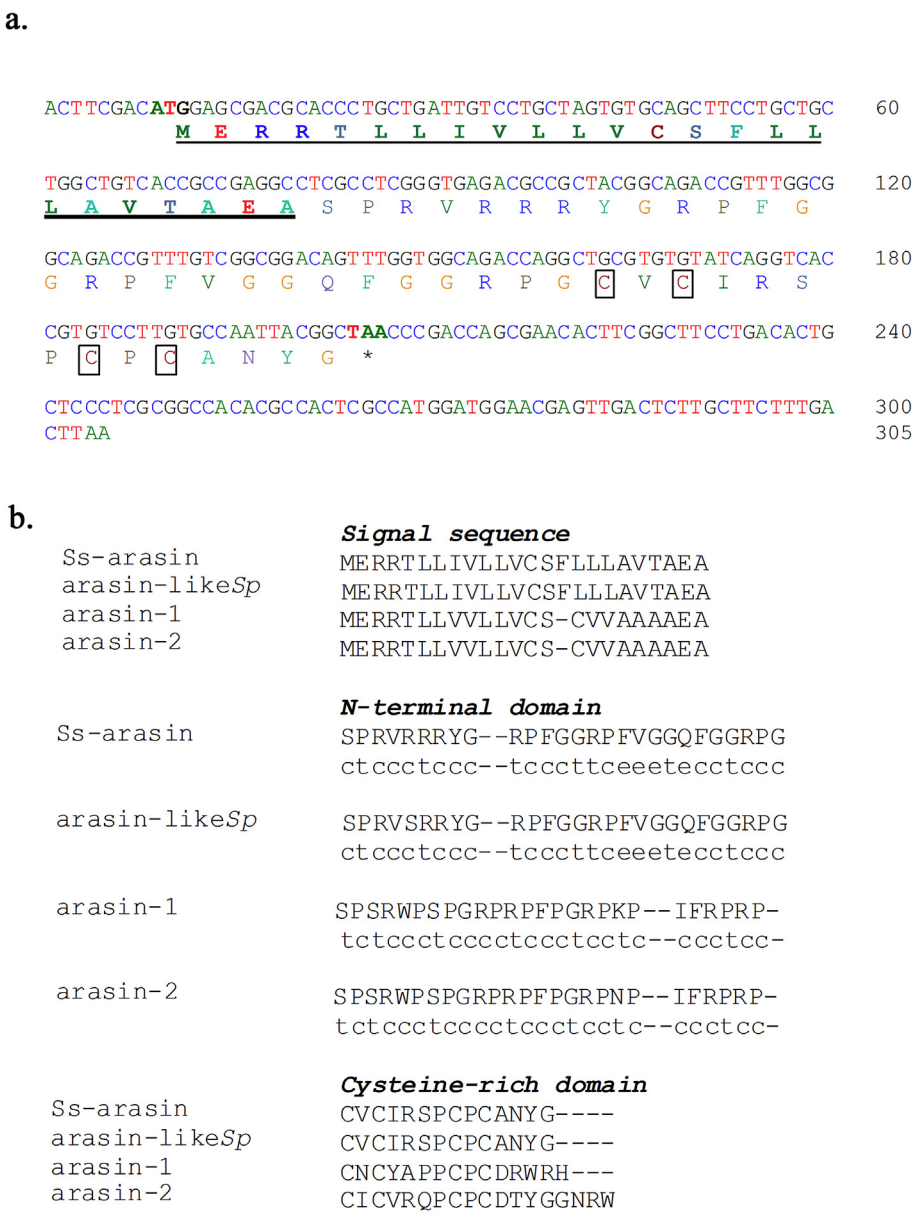

Fig. 1. Nucleotide and deduced amino acid sequences of Ss-arasin. a). Nucleotide numbers are shown on the right. The start codon is in bold and the termination codon is indicated with asterisk (*). The predicted signal peptide sequence is underlined. Four conserved cysteine residues are in box. **b)** Multiple alignment of the deduced amino acid sequence of Ss-arasin (MK044533) from mud crab, *Scylla serrata* with *Scylla paramamosain,* arasin-like*Sp* (ADT71678), arasin-1 (DQ859904) and arasin 2 (DQ859905) from the spider crab, *Hyas araneus*. Secondary structure predictions of the N-terminal domain are shown in lower cast letter: t = reverse turn, c = random coil and e = extended strand.

show that *H. araneus* arasin-1 and 2 are Arg/Pro-rich whereas *S. paramamosain,* arasin-like*Sp* and Ss-arasin are Arg/Gly rich. The ratio of Arg/Pro/Gly in arasin-1 is 6:9:2 compared to 7:4:8 in Ss-arasin. Because proline is sterically the most restrictive amino acid and glycine the most flexible residue, one could expect differences in the peptide secondary structures between Ss-arasin and arasin-like*Sp*.

To evaluate the secondary structure of Ss-arasin we used the Chou and Fasman algorithm [13] and compared the outcome using the same algorithm for the corresponding sequences of arasin-like*Sp* and *H. araneus* arasin-1 and 2. In the absence of disulfide bonds the prediction of the cysteine-rich region is less reliable; therefore we focused on the N-terminal domain only. The predicted secondary structures of Ss-arasin and arasin-like*Sp* was identical and comprised random coil with an extended strand of the sequence FVGGQ. Seven turns were predicted at glycine or proline residues (Fig. 1b). The secondary structures predicted for arasin-1 and -2 showed less secondary structures than Ss-arasin. Only random coil and seven turns were predicted, with three of the turns in positions identical to arasin-like*Sp*. As reported by Paulsen et al. the unstructured character of its N-terminal active segment was verified by circular dichroism spectroscopy [14]. Whether or not the N-terminal segment is also the active region of Ss-arasin and arasin-like*Sp* needs to be investigated.

Antimicrobial polypeptides with multi-domain structure comprising a Gly rich N-terminal domain and a cysteine-rich C-terminal domain have been isolated from various arthropod species [15]. For instance, Type II crustins cationic in nature with 70–170 amino acid residues have been isolated from crustaceans. The N-terminal glycine-rich domain is followed by a cysteine-rich domain which is further C-terminally extended by a whey acidic protein (WAP). Type II crustin is active against Gram-positive bacteria [16,17]. Hyastatin, is comprised of three domains, the glycine-rich N-terminal domain, the Cys-containing C-terminal domain and a short arginine/proline-rich segment intercepting the two. Hyastatins show anti-microbial activity against Gram-positive and Gram-negative bacteria [18]. The first combination of a highly positively charged Arg/Gly rich domain in combination with a C-terminal cysteine-rich domain was reported for the arasin-*likeSp* isolated from mud crab, *S. paramamosain*, arasin-*likeSp* shows anti-microbial activity against Gram-positive and Gram-negative bacteria [11].

3.2. Gene expression analysis in response to LPS challenge

Real-time-PCR in haemocytes of mud crab, *S. serrata* was conducted

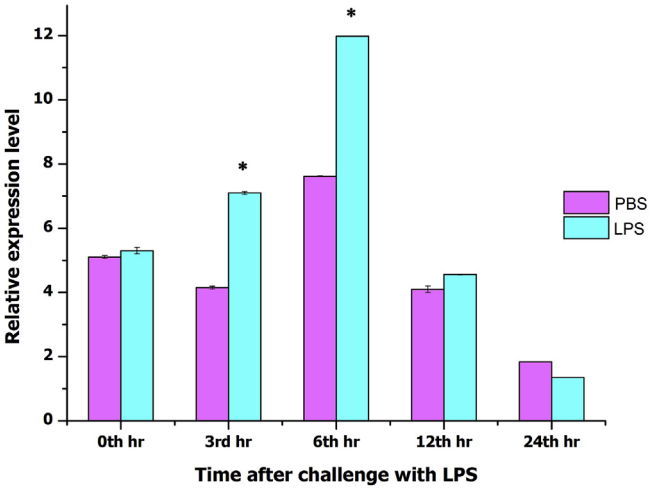

Fig. 2. Temporal expression pattern of Ss-arasin after challenge with LPS. Significant differences between the test group and control group are indicated with (*) for $p < 0.05$.

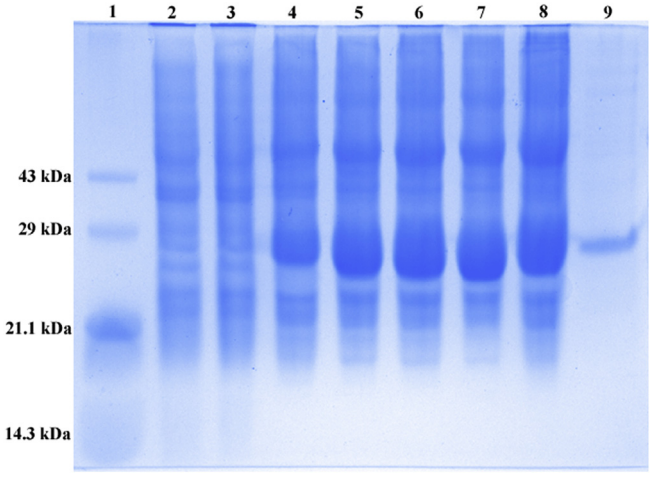

Fig. 3. Time-course of rSs-arasin expression in *Escherichia coli*. Tricine SDS-PAGE analysis before and after IPTG induction. Lane 1:Mid-range protein ladder; Lane 2: control prior to IPTG induction; Lane 3–8: cells after 0–5 h post IPTG induction.; Lane 9: purified recombinant rTrx-hexaHis-Ss-arasin (26.2 kDa).

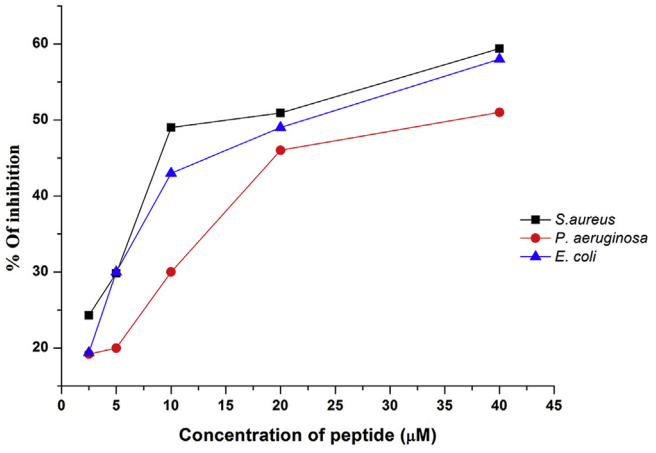

Fig. 4. Antimicrobial activity. Antimicrobial activity of rSs-arasin against *Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli* at various concentrations.

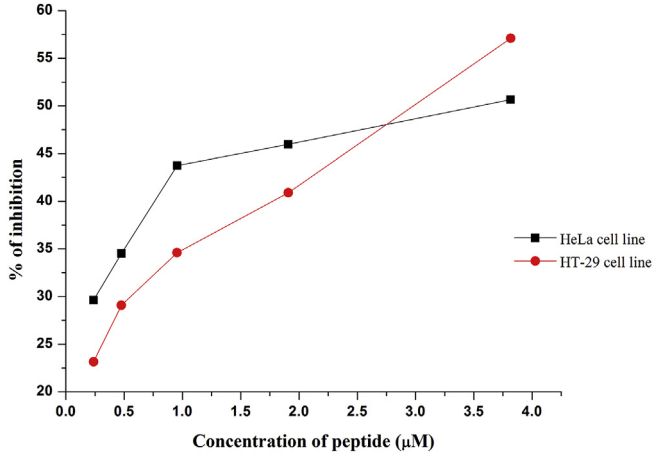

Fig. 5. Cytotoxicity analysis. *In vitro* cytotoxicity of the recombinant rSs-arasin in HT-29 cell lines and HeLa cell lines at various concentrations.

after challenge with LPS and compared with the PBS control group. The housekeeping gene β-actin was used to normalize gene expression. LPS stimulation was significantly upregulated from 3rd hour onwards (Fig. 2). The mRNA expression of Ss-arasin reached maximum at 6th hour and afterwards decreased over time. At maximum the mRNA level of Ss-arasin increased 4-fold over control. Similar results were reported on arasin-like*Sp* species *S. paramamosain,* where mRNA expression was upregulated at 3 h post-bacterial challenge [11]. As shown in Fig. 2 Ss-arasin early response indicated that it is a constitutive and inducible gene maintaining a constant level in the animal for immediate detection of an infectious invader.

3.3. Recombinant expression and production of Trx-hexaHis-arasin fusion protein (= rSs-arasin)

To further determine whether the purified Ss-arasin protein has antibacterial and anticancer activity, Ss-arasin was over-expressed in *E. coli*. The resulting fusion protein was purified on a Ni-NTA column. SDS-PAGE analysis showed a single band for the highly pure fusion protein at 26.2 kDa (combined molecular weight of Ss-arasin (7 kDa) and the fusion tags (19.2 kDa) (Fig. 3).

3.4. Antimicrobial activity

Antimicrobial activity of the purified and refolded rTrx-hexaHis-Ss-arasin was tested for the ability to inhibit the proliferation of micro-organisms by determining the minimum inhibitory concentration (MIC). The protein expression control, rTrx-hexaHis was used as the negative control in the assays. The resulting activities for rSs-arasin represent the activity measured for rTrx-hexaHis-Ss-arasin minus the activity of rTrx-hexaHis. The assay performed with two-fold serial dilutions of rSs-arasin against Gram-positive *S. aureus* showed a gradual increase of inhibition over the full range reaching a maximum of 59.4% inhibition at 40 mM concentration (Fig. 4). Growth of Gram-negative *P. aeruginosa* and *E. coli* was inhibited by 51% and 58% only at 40 mM concentration. In earlier reports arasin-like*Sp* from *S. paramamosain* showed antimicrobial activity against Gram-positive *Staphylococcus haemolyticus, Aerococcus viridans*, *Micrococcus luteus* and *Bacillus subtilis*, and the Gram-negative *Vibrio harveyi* and *Vibrio anguillarum* bacteria [11]. Similarly, arasin-1 from *H. araneus* displayed antimicrobial activity against both Gram-positive (*Corynebacterium glutamicum* and *S. aureus*) and Gram-negative (*V. anguillarum* and *E. coli*) bacteria [7]. More significantly, it was noted that the broad spectrum of activity of rSs-arasin to be an efficient therapeutic agent against multidrug resistant strains.

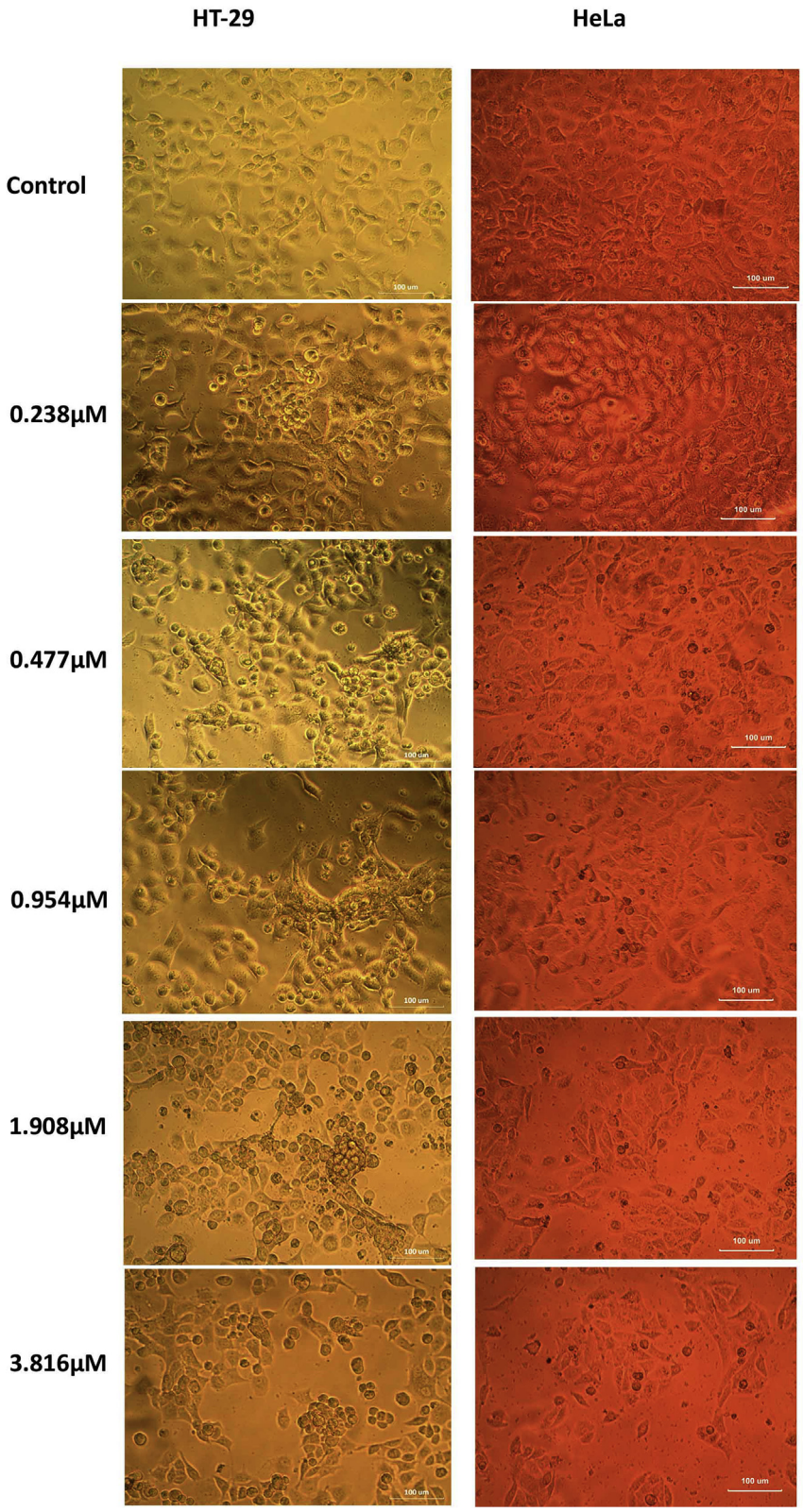

Fig. 6. Morphology of HT-29 and HeLa cell lines under phase contrast microscopy. Morphology of HT-29 and HeLa cell lines before and after treatment with various concentrations of rSs-arasin observed under phase contrast microscopy (Magnification ×100).

3.5. *In vitro cytotoxicity*

The MTT assay was employed to determine cytotoxicity of rSs-arasin in two cancer mammalian (HT-29 and HeLa) cell lines with an IC_{50} of 2.90 µM for HT-29 cells and 3.06 µM for HeLa cells. At higher concentration of rSs-arasin, the peptide exhibited 57% and 50% growth reduction in HT-29 and HeLa cells, respectively (Fig. 5). However, this study clearly implies that rSs-arasin is involved in anticancer activity; further molecular studies are required to characterize the mechanisms of cytotoxicity of rSs-arasin.

3.6. Conclusion

In conclusion, in the present study, we showed antimicrobial and anticancer activities of rSs-arasin. However, further studies need to be carried out to generate recombinant peptide mutants to maximize its efficacy. Furthermore, the antimicrobial and anticancer activity against clinical isolates and carcinoma cell lines need to be investigated to manifest potential therapeutic applications of this recombinant peptide. Therefore, rSs-arasin is a promising molecule to develop new biogenic drugs to overcome the current bottleneck generated by the drug resistant pathogens and as a chemotherapeutic drug in combating cancer. Antibacterial activity of rSs-arasin would hopefully use as therapeutic or prophylactic agent for disease control and health management in crab aquaculture in the near future.

Acknowledgments

This work was financially supported by UGC-Dr. D.S. Kothari postdoctoral fellowship programme. The authors wish to thank to the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology for the facilities. The authors gratefully acknowledge the colleagues of our department for their support during this work.

References

[1] V.D. Duin, D. Paterson, Multidrug resistant bacteria in the community: trends and lessons learned, Infect. Dis. Clin. N. Am. 30 (2) (2017) 377–390.
[2] P. Bulet, R. Stocklin, L. Menin, Anti-microbial peptides: from invertebrates to vertebrates, Immunol. Rev. 198 (2004) 169–184.
[3] S.H. Marshall, G. Arenas, Antimicrobial peptides: a natural alternative to chemical antibiotics and a potential for applied biotechnology, Electron. J. Biotechnol. 6 (2003) 271–284.
[4] Y. Gueguen, J. Garnier, L. Robert, M.P. Lefranc, I. Mougenot, J. de Lorgeril, M.G. Janech, P. Gross, G. Warr, B. Cuthbertson, M.A. Barracco, P. Barracco, A. Aumaleas, A. Et, PenBase, the shrimp antimicrobial peptide penaeidin database: sequence-based classification and recommended nomenclature, Dev. Comp. Immunol. 30 (2006) 283–288, https://doi.org/10.1016/j.dci.2005.04.003.
[5] J.M. Relf, J.R.S. Chisholm, G.D. Kemp, V.J. Smith, Purification and characterisation of a cysteine-rich 11.5 kDa antibacterial peptide from the granular haemocytes of the shore crab, *Carcinus maenas*, Eur. J. Biochem. 264 (1999) 1–9.
[6] C. Mu, P. Zheng, J. Zhao, L. Wang, H. Zhang, L. Qiu, et al., Molecular characterization and expression of a crustin-like gene from Chinese mitten crab, *Eriocheir sinensis*, Dev. Comp. Immunol. 34 (2010) 734–740.
[7] K. Stensvag, T. Haug, S.V. Sperstad, O. Rekdal, B. Indrevoll, O.B. Styrvold, Arasin 1, a proline-arginine-rich antimicrobial peptide isolated from the spider crab, *Hyas araneus*, Dev. Comp. Immunol. 32 (2008) 275–285.
[8] D.X. Zhao, S.H. Song, Q. Wang, X.W. Zhang, S.N. Hu, L.Q. Chen, Discovery of immune-related genes in Chinese mitten crab (*Eriocheir sinensis*) by expressed sequence tag analysis of haemocytes, Aquaculture 287 (2009) 297–303.
[9] C. Imjongjirak, P. Amparyup, A. Tassanakajon, S. Sittipraneed, Anti- lipopolysaccharide factor (ALF) of mud crab Scylla paramamosain: molecular cloning, genomic organization and the antimicrobial activity of its synthetic LPS binding domain, Mol. Immunol. 44 (2007) 3195–3203.
[10] C. Imjongjirak, P. Amparyup, A. Tassanakajon, S. Sittipraneed, Molecular cloning and characterization of crustin from mud crab Scylla paramamosain, Mol. Biol. Rep. 36 (5) (2009) 841–850.
[11] C. Imjongjirak, P. Amparyup, A. Tassanakajon, Two novel antimicrobial peptides, arasin-like*Sp* and GRP*Sp*, from the mud crab *Scylla paramamosain*, exhibit the activity against some crustacean pathogenic bacteria, Fish Shellfish Immunol. 30 (2011) 706–712.
[12] L.B. Talarico, R.G. Zibetti, P.C. Farıa, L.A. Scolaro, M.E. Duarte, M.D. Noseda, C.A. Pujol, E.B. Damonte, Anti-herpes simplex virus activity of sulfated galactans from the red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemia crenulata*, Int. J. Biol. Macromol. 34 (2004) 63–71.
[13] T. Ashok Kumar, CFSSP: Chou and fasman secondary structure prediction server, Wide Spectr. 1 (9) (2013) 15–19.
[14] V.S. Paulsen, H.M. Blencke, M. Benincasa, T. Haug, J.J. Eksteen, O.B. Styrvold, M. Scocchi, K. Stensvag, Structure-activity relationships of the antimicrobial peptide arasin 1 — and mode of action studies of the N-terminal, proline-rich region, PLoS One 8 (1) (2013) 1–11.
[15] P. Bulet, C. Hetru, J.L. Dimarcq, D. Hoffmann, Antimicrobial peptides in insects; structure and function, Dev. Comp. Immunol. 23 (1999) 329–344.
[16] V.J. Smith, J.M. Fernandes, G.D. Kemp, C. Hauton, Crustins: enigmatic WAP domain-containing antibacterial proteins from crustaceans, Dev. Comp. Immunol. 32 (2008) 758–772.
[17] A. Tassanakajon, P. Amparyup, K. Somboonwiwat, P. Supungul, Cationic antimicrobial peptides in penaeid shrimp, Mar. Biotechnol. 12 (2010) 487–505.
[18] S.V. Sperstad, T. Haug, T. Vasskog, K. Stensvag, Hyastatin, a glycine-rich multidomain antimicrobial peptide isolated from the spider crab (*Hyas araneus*) hemocytes, Mol. Immunol. 46 (2009) 2604–2612.