



Therapeutic Cationic Antimicrobial Peptide (CAP) Derived from Fish Aspartic Proteinase Cathepsin D and its Antimicrobial Mechanism

Akila Sathyamoorthi^{1,2} · Venkatesh Kumaresan¹ · Rajesh Palanisamy¹ · Mukesh Pasupuleti³ · Mariadhas Valan Arasu⁴ · Naif Abdullah Al-Dhabi⁴ · Kasi Marimuthu⁵ · S. M. Nurul Amin⁶ · Aziz Arshad^{6,7} · Fatimah Md. Yusoff^{6,7} · Jesu Arockiaraj^{1,7}

Accepted: 24 November 2017 / Published online: 28 November 2017
© Springer Science+Business Media, LLC, part of Springer Nature 2017

Abstract

Aquatic organisms are rich in antimicrobial peptides which play key role against pathogens during infections. Cathepsin is one of immune proteases which have been proven with multiple functions including antimicrobial activity, but their role as antimicrobial peptides has not been elucidated so far in aquatic organisms. This study reports the identification and characterization of antimicrobial peptides from fish cathepsin D. *Channa striatus* (Cs) cathepsin D (Cath D) was identified from its established cDNA library. Multiple sequence alignment was performed to analyze the homology of CsCathD with other cathepsin. Based on the amino acid propensity scale, two putative antimicrobial regions were identified, synthesized and analyzed for their antimicrobial potency. Gene expression of CsCath D and its mRNA pattern upon pathogenic infection was also observed using real time PCR. All the bioinformatics analysis indicated the gene specific characteristic features of CsCath D. CsCathD mRNA expression was highly expressed at 24 h for bacteria (*Aeromonas hydrophila*) and 48 h for fungus (*Aphanomyces invadans*). The CsCath D derived CAPs namely, PL12 and NM12 showed their commendable inhibition towards *Bacillus mycoides* of the tested bacteria. The cell membrane disruption was observed with PL12 against *B. mycoides* in flow cytometer. With all proceedings, it is possible to conclude that CsCathD might be a potent immuno modulator and the reported CAPs could be developed as therapeutic agents to treat bacterial pathogenic infections.

Keyword Cationic anti-microbial peptide · Fish · Gene expression · Cell membrane disruption · Flow cytometry

Abbreviations

Cath D	Cathepsin D	CAP	Cationic antimicrobial peptides
Cs	<i>Channa striatus</i>	AMP	Antimicrobial peptides
ORF	Open reading frame	EUS	Epizootic ulcerative syndrome
		MIC	Minimum inhibitory concentration
		RBC	Red blood cells
		qRT PCR	Quantitative real time polymerase chain reaction

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10989-017-9652-y>) contains supplementary material, which is available to authorized users.

✉ Jesu Arockiaraj
jesuaraj@hotmail.com

¹ Division of Fisheries Biotechnology & Molecular Biology, Department of Biotechnology, Faculty of Science and Humanities, SRM University, Kattankulathur, Chennai, Tamil Nadu 603 203, India

² Department of Biotechnology, SRM Arts & Science College, Kattankulathur, Chennai 603 203, India

³ Lab PCN 206, Microbiology Division, CSIR-Central Drug Research Institute, B.S. 10/1, Sector 10, Jankipuram Extension, Sitapur Road, Lucknow, Uttar Pradesh 226 031, India

⁴ Department of Botany and Microbiology, Addiriyah Chair for Environmental Studies, College of Science, King Saud University, P. O. Box 2455, Riyadh 11451, Saudi Arabia

⁵ Department of Biotechnology, Faculty of Applied Sciences, AIMST University, 08100 Bedong, Kedah Darul Aman, Malaysia

⁶ Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

⁷ Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia

ATCC American type culture collection
MTCC Microbial type culture collection

Introduction

During infections, vertebrate species protects themselves by both innate and adaptive responses; however, the antibody and specific cell-mediated immune responses are poorly developed in primitive vertebrates such as fish (Ellis 1982). Therefore, there is a substantial dependence on innate immune response for protection against microbial invasion (Magnadottir 2006). Among them, cationic antimicrobial peptides (CAPs) have been recognized as one of the major defense molecules which act against infections especially in aquatic organisms. Due to its proficient bactericidal action and lesser chance to develop resistance against them, CAPs have become attractive and suitable candidates for clinical use (Patrzykat et al. 2001).

Antimicrobial peptides (AMPs) are generally short peptides that are derived from proteins performing wide variety of functions. AMPs comprises common features including positive charge, hydrophobic or amphipathic nature that allow them to interact with negatively charged phospholipids head groups and hydrophobic fatty acid chains of microbial membranes, resulting in the formation of pores on the microbial membrane. AMPs have been isolated from a wide range of animals and plant species (Hancock and Lehrer 1998), including fish (Cole et al. 1997; Patrzykat et al. 2001) which exhibit multiple functions like broad-spectrum antimicrobial activity as well as immuno-modulatory functions (Boman 1995). Though several AMPs have been isolated from fish (Cole et al. 1997; Park et al. 1997, 1998; Lauth et al. 2002; Shike et al. 2002; Cho et al. 2002a, b), their numbers are very less when compared to the peptides isolated from other species. This is because of the difficulty in isolating functionally active CAPs from fish tissues. Several reports stated that most AMPs are derived from a biologically inactive pro-protein with antimicrobial activities such as histones (Cho et al. 2002a, b) and are released when they are processed to their active form. These peptides can either function intracellular in circulating leukocytes or in the external environment after released by secretory cells and other granulated epithelia (Hancock and Scott 2000). Fish mucus is generally rich in secreted antimicrobial peptides which act in extracellular environment (Palanisamy et al. 2016). Recently, few studies have reported the activity of peptides that are derived from immune proteins which has the advantage of scale up of production at industrial level (Kumaresan et al. 2015a, b).

Cathepsins are lysosomal proteolytic enzymes which catalyzes the hydrolysis of variety of protein substrates. Based on their active residues, proteases are classified as serine protease, cysteine protease and aspartate proteases where

cathepsins are further classified into 12 types according to its substrate specificity. For instance, cathepsin A and G are serine proteases; cathepsin B, C, F, H, K, L1, L2, O, S, W, and Z are cysteine proteases, whereas cathepsin D and E are aspartic proteases. In aspartic proteases, aspartic acid residue acts as catalytic residue which involved in the catalysis of the peptide substrate and functions chiefly in the degradation of intracellular and endocytosed proteins. Cathepsin D is one of the major aspartic protease of the pepsin super family, which plays an important role in lysosome (Lee et al. 2007; Jia and Zhang 2009).

Cathepsin D is a 40 kDa protein with an isoelectric point of 6.95, whereas cathepsin E is a dimeric peptide each having a molecular mass of 40 kDa with an isoelectric point of 4.6 (Lapresle et al. 1986). Furthermore, cathepsin D plays an important role in the lysosomal-mediated degradation of proteins (Tang and Wong 1987). It has broad peptide bond specificity similar to pepsin and has been shown to be involved in various physiological pathways, such as intracellular catabolic proteolysis (Baricos et al. 1987), extracellular proteolysis and processing, secretion and activation of enzymes and hormones (Baldocchi et al. 1993). Regarding antimicrobial function of cathepsin D, it cleaves the Ser¹⁹–Arg²⁰ bond of histone H2A to produce parasin I (Cho et al. 2002a, b). Also, CathD has also involved in cleavage of dermicidin, an antimicrobial peptide present in human skin (Baechle et al. 2006). However, the direct antimicrobial role of the whole protein or derived peptides from cathepsin D has not been elucidated so far.

Cathepsins are multifunctional proteases which are involved in various immune processes including antimicrobial activity. Lysosomal cathepsin from human neutrophils is a chymotrypsin-like protease which also possesses antimicrobial activity. The antimicrobial activity, however, is independent of protease activity, because treatment of this enzyme with the irreversible serine protease inhibitor diisopropyl fluorophosphate has no effect on its antimicrobial action. Therefore, they serve as a unique candidate for the antimicrobial peptide selection. Though the antimicrobial activity of cathepsin has been studied extensively, the antimicrobial activity of a specific region, in other words, the antimicrobial activity of the short peptide derived from cathepsin has not been elucidated.

Channa striatus also called striped murrel, has been considered as food fish in many parts of India (Jayaram 1981). Besides the high quality of their flesh in terms of taste and texture, they also have good market value due to the low fat, fewer intramuscular spines and medicinal qualities (Arockiaraj et al. 2004). Epizootic ulcerative syndrome (EUS) is a malady among wild and cultured freshwater fish, especially *C. striatus* which is caused by fungus *Aphanomyces invadans* which further attracts other pathogens that increases the severity of the infection (Lilley et al. 1998).

Considering the above problems, the present study focuses on the expression of cathepsin D from *C. striatus* during infection by bacteria, fungi and viral analogue and to isolate new intoxicating antimicrobial peptides (AMPs) from cathepsin D (Cath D) of *C. striatus* against pathogens.

In this study, a molecular analysis of cathepsin D from *C. striatus* (Cs) was reported in detail at molecular level including bioinformatics analysis and the activity of therapeutic cationic antimicrobial peptide (CAP) derived from cathepsin D. In brief, two peptide regions with reputed cationic hydrophobic antimicrobial activity from CsCathD were identified. Both the peptides were synthesized and antibacterial activity was analyzed against a wide range of bacterial species; and minimum inhibitory concentration (MIC) of the peptides was obtained. Cytotoxicity assay was performed to confirm the non-toxicity nature of peptides to human RBC. The antibacterial activity of the peptides was determined by dye exclusion assay which exhibited the bacterial cell membrane disruption ability of the peptides; thus observed it by flow cytometer analysis. Further, to reveal the expression pattern of CsCathD transcripts in various tissues of *C. striatus* at the time of bacterial, fungal and poly I:C infection, a qRT PCR was conducted.

Materials and Methods

Fish

Channa striatus (52 ± 7 g) were collected from Porur Lake (13.034223°N and 80.15065°E), Chennai, Tamil Nadu, India and transported to the aquarium at the research laboratory in plastic containers. The fishes were maintained, reared and fed in the laboratory as the information given in our earlier studies (Arasu et al. 2016; Kumaresan et al. 2016). Then, the fishes were challenged with pathogens.

Pathogens

Aeromonas hydrophila (MTCC#1739), a Gram-negative bacterium which causes EUS in fish was purchased from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India. Overnight grown bacterial culture was inoculated in fresh Nutrient Broth Medium (HiMedia Labs, India) and allowed to grow at 37 °C for 4–6 h to ensure that the cells are in exponential growth phase, then centrifuged at 5000 RPM for 5 min and adjusted to 10^6 CFU/ml using PBS. *Aphanomyces invadans* is an oomycete which is the primary causative agent of EUS, isolated from EUS infected *C. striatus*. The fungus was grown in GP broth at 23 °C for 4 days and the spores were collected. The spores were counted using haemocytometer and adjusted to 10^2 spores/ml. The concentration of the bacterial cells and fungal spores and

their growth media were optimized in our previous studies (Arockiaraj et al. 2015).

For the antimicrobial activity, nine bacterial pathogens were used which are purchased from MTCC and American type culture collection (ATCC). The strains are *Bacillus cereus* ATCC 2106, *B. mycoides* MTCC 8920, *B. subtilis* ATCC 6051, *Escherichia coli* ATCC 25922, *E. coli* ATCC 9637, *Klebsiella pneumoniae* ATCC 27736, *S. aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 25668 and *Micrococcus luteus* MTCC 6164. The cultures were grown as suggested by the supplier. All the strains were stored at -70 °C until they were thawed for use, sub-cultured daily and cultured to optimal concentrations before the antimicrobial assays.

Pathogenic Challenge and Tissue Collection

After a week period of acclimatization, the fishes were challenged with pathogenic fungus, bacteria and immune stimulant of viral residue. A batch of 15 individuals for each challenge was experimented. The disease was challenged by intraperitoneal injection at the concentration of 10^2 spores (fungus), 10^6 cells (bacterial) and 100 µg (poly I:C). Similar quantity of 1X PBS was given to the control individuals.

The various tissues including head kidney, kidney, intestine, liver, skin, gills, spleen, brain, heart, muscle and blood were collected from *C. striatus* by sacrificing the fish at 0, 3, 6, 12, 24, 48 and 72 h post-injection time. The dissected tissues were flash frozen immediately in liquid nitrogen and then stored at -80 °C until the total RNA extracted. A maximum of 10 fish per tank were maintained during the experiment and for each challenge of each time point three fishes were sampled.

RNA and cDNA Synthesis

Total RNA was isolated from the collected tissues of *C. striatus* using High Pure RNA Tissue kit (Roche Diagnostics GmbH, Germany). Further, the cDNA was produced from the RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Germany). The quality and quantity of both the RNA and cDNA was analysed in UV-1800 Spectrophotometer (Shimadzu Corp, Japan).

Identification of CsCathD

Full length CsCathD was identified from the constructed cDNA library of *C. striatus* that established using GS FLX™ technology. In short, cDNA library of *C. striatus* was developed using total RNA obtained from *C. striatus*. From the total RNA, mRNA was further purified (Miltenyi Biotech, Germany) and then cDNA (Invitrogen, USA) was synthesized. The cDNA library was developed using

Invitrogen (USA) kit and sequenced using GS FLX™ technology (Roche, India) as described in the company protocol. The detailed protocol of cDNA library development was reported in our earlier studies (Arockiaraj et al. 2015; Kumaresan et al. 2015a, b). During BLAST search (<http://blast.ncbi.nlm.nih.gov/Blast>), CsCathD transcript was identified from the library and considered for the present study. The identified cDNA sequence has been submitted to European Nucleotide Archive, EMBL Bank.

Bioinformatics Analysis

A putative open reading frame (ORF) and its corresponding amino acid sequence of CsCathD cDNA were determined using ExPasy Translate Tool (<http://web.expasy.org/translate>). The physico-chemical properties of the CsCathD polypeptide including number of amino acid residues, molecular weight, iso-electric point (pI), instability index and aliphatic index was analyzed using PROTPARAM online server (<http://www.expasy.org/tool/protparam>). Also, the CsCathD was subjected to similarity search, which was performed through BLAST at NCBI Database (<http://blast.ncbi.nlm.nih.gov/Blast>). The analysis of secondary structural elements of CsHSC70 was conducted in SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html). The motifs and domains of the CsCathD from the conserved sequence homology were identified from PROSITE (<http://prosite.expasy.org/scanprosite/>). The putative cleavage site of the signal peptide was predicted by SignalP (<http://www.cbs.dtu.dk/services/SignalP/>). Transmembrane segments of the CsCathD protein was predicted using SACS MEMSAT2 tool (<http://www.sacs.ucsf.edu/cgi-bin/memsat.py>). To identify the identical and similar residues of CsCathD with homologous sequences, pairwise and global sequence alignments were carried out using ClustalW multiple alignment algorithms in Bio Edit (ver. 7.1.3.0). The evolutionary distance of CsCathD with other related sequences was obtained from Poisson model phylogenetic tree construction using neighbor joining (NJ) algorithm in MEGA 5.05 with 1000 bootstrap repetitions. Three dimensional structures of CsCathD protein was predicted by I-TASSER server. The structure was viewed and analyzed using PyMol tool (Version 0.99).

Antimicrobial Peptide Synthesis

CsCathD derived peptides, PL12 and NM12 (Table 1) was synthesized by solid phase peptide synthesis method (SynPeptide Co. LTD., Shanghai, China). The peptides were further purified in reverse phase high-performance liquid chromatography (HPLC) and the purity of the peptides was confirmed. The molecular mass of the purified peptide analogs were confirmed by MALDI TOF-MS. The

Table 1 Amino acid sequences, chemical formula and molecular mass of PL12 and NM12 peptides of CsCathD

Peptides	Amino acid sequences	Chemical formula	Mol. wt. (Da)
PL12	PLKKFRSIRREL	C ₇₀ H ₁₂₃ N ₂₃ O ₁₆	1542.88
NM12	NITRQAYWQIHM	C ₇₀ H ₁₀₅ N ₂₁ O ₁₈ S ₁	1560.79

chemical nature of peptides was analyzed by a peptide calculator (http://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php).

In Vitro Antibacterial Activity of the Peptides

The MIC of each peptide was determined using a broth micro dilution assay (Wu and Hancock 1999; Ravichandran et al. 2016). Briefly, the serial dilutions of the peptides were made in PBS in 96-well polypropylene micro titer plates. The above listed bacterial strains were grown overnight to the mid-logarithmic phase and diluted to give a final inoculum size of 5×10^5 CFU/ml. A suspension of 15 μ l bacteria was added to each well of a 96-well plate, and the plate was incubated overnight at the appropriate temperature. Inhibition of growth was determined by measuring the absorbance at 595 nm with a microplate reader, after an incubation time of 18 ± 2 h at 37 °C. Inhibition was defined as growth less than or equal to one-half of the growth observed in control wells to which no peptide was added.

In Vitro Hemolysis of Peptide Using Human RBC

The hemolytic activity of the peptides (Ravichandran et al. 2016) was verified against human red blood cells (hRBC). In brief, fresh hRBC with EDTA (2 mg/ml) were rinsed three times with PBS by centrifugation at 800 g for 10 min and re-suspended in PBS. Peptide (conc. 600 μ M) dissolved in PBS was then added to 50 μ l of a solution of the stock hRBC in PBS to reach a final volume of 100 μ l (final hRBC conc. 5% v/v). The resulting suspension was incubated at 37 °C for 1 h. The samples were then centrifuged at 800 g for 10 min. The supernatant was removed and the liberated haemoglobin was monitored by measuring the absorbance of the supernatant at 540 nm. Controls for zero haemolysis (blank) and 100% haemolysis consisted of hRBC suspended in PBS and Triton 100-X 0.2%, respectively.

Bacterial Cell Membrane Disruption by PL12

The activity of PL12 against *B. mycoides* membrane is assessed by dye exclusion assay using propidium iodide (PI) (Ravichandran et al. 2016). This dye binds to double stranded DNA by intercalating between base pairs when the bacterial cell membrane is compromised or cleaved. The

protocol was followed and standardized for the staining of non-viable cells. Briefly, the bacterial cells were grown to the mid-logarithmic phase and diluted to give suspension of 1×10^6 CFU/ml in 200 μ l of 10 mM PBS at pH 7.4 and incubated at 37 °C for 180 min with PL12 along with controls without peptide PL12. After incubation the cells were harvested by centrifugation at 1700 g for 15 min at 4 °C. The cells were subjected to 2 microliters of PI (5 μ g/ml) for 10 min at room temperature and mix well. The samples are protected from light penetration until analysis on the flow cytometer.

Gene Expression

Relative *CsCathD* expression was performed using β -actin as housekeeping molecule in real time PCR (Light Cycler 96 Real Time PCR, Roche Diagnostics GmbH, Germany). The assay was performed with the following materials: 2 μ l cDNA sample, 10 μ l Fast SYBR® Green Master Mix, 0.5 μ l PCR forward/reverse primers (10 mM) and 7 μ l nuclease-free water. The thermal profile was set as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s and 58 °C for 60 s. The results were calculated using $2^{-\Delta\Delta CT}$ method as we (Arockiaraj et al. 2004; Palanisamy et al. 2016; Arasu et al. 2016) described in our earlier study. The primers used for the assay as follows: *CsCath D* F1: GAT GGT GTG GCT CCA GTA TTT (Forward Primer), *CsCath D* R2: GAA CAG GAA CCC AGA TAC CAC (Reverse Primer), β -actin F3: TCT TCC AGC CTT CCT TCC TTG GTA (Forward Primer) and β -actin R4: GAC GTC GCA CTT CAT GAT GCT GTT (Reverse Primer). The house keeping molecule's primer designed from GenBank Accession ID EU570219.

Statistics

Statistical performance of relative gene expression, hemolysis and MIC was analysed on SPSS (ver. 11.5) using one-way ANOVA and post-ANOVA Tukey's Multiple Range Test.

Results

CsCathD cDNA Characterization

A full length cDNA sequence of cathepsin D was identified from previously constructed cDNA library of *C. striatus*. The reported nucleotide sequence of *CsCathD* can be accessed from NCBI database using the accession number, HF94729. The complete *CsCathD* cDNA sequence is 1191 bp with an open reading frame (ORF) of 1188 bp which specifies a protein of 396 amino acids and with predicted MW 43.07 kDa having the isoelectric point (pI) of

6.60, showing the pH at which a particular molecule carries no net electrical charge. The protein is made up of 35 negatively charged residues (Asp and Glu) and 34 positively charged residues (Arg and Lys), with an instability index of 39.46, which classifies the protein as stable. The amino acid sequence has a putative signal peptide at the N terminal, starting from M¹ to A¹⁷ having cleavage site between A¹⁷ and A¹⁸ residues. *CsCathD* has 3 strong transmembrane helices at Phe⁴–Pro²³ (20 residues), Tyr¹²⁸–Ser¹⁴⁹ (22 residues) and Ile³⁶¹–Phe³⁸³ (23 residues) asserting that the region bind to signal molecules in the extra cellular space and generate different intra cellular signals on the opposite side of the plasma membrane.

Homology, Domain and Motif Analysis

The BLAST analysis for *CsCathD* amino acid sequence had a similarity of 90% with *Larimichthys crocea* (Large yellow croaker), 89% with *Lates calcarifier* (Asian sea bass) and 88% with *Oryzias latipes* (Japanese rice fish) and the sequence also had similarities with green sea turtle, birds, chicken, street rat and monkey. The conserved protein domain family illustrates that *CsCathD* amino acid sequence has a peptidase A₁ domain region placed in between Y⁷⁶ and A³⁹³. This domain has three active site regions namely catalytic residue, catalytic motif and active flap site. The important feature of *CsCathD* is the presence of conserved catalytic residues D²⁸, D⁹⁴ (data shown in Fig. 1 of E-Suppl. File) among fishes, frog, cattle, chicken and human. The catalytic motif active site of *CsCath D* is present at N (D³⁹, S⁴⁰, G⁴¹, S⁴²) and C-terminal (D²⁸¹, T²⁸², G²⁸³, T²⁸⁴) lobes of the peptidase family (data shown in Fig. 2 of E-Suppl. File). Similarly these residues are conserved among zebra fish, frog, chicken and human. The third region active flap site is an extended loop projecting over the cleft to form an 11 residue flap enclosing within the active site. The active flap residues of *CsCathD* viz., A¹³⁷–G¹⁴⁷ replaced few residues after the first catalytic residue D²⁸. Scan prosite analysis showed disulphide bridge between C³¹⁵–X*–C³⁵² (C–X*–C) and indicated the presence of six high possibility motifs viz., N-myristolation pattern (7 sites), protein kinase C phosphorylation pattern (4 sites), casein kinase II phosphorylation (4 sites), tyrosine kinase phosphorylation pattern (1 site), N glycosylation pattern (2 sites) and leucine zipper pattern (1 site).

Structure Analysis

The secondary structural feature analysis of *CsCathD* using polyview tool predicted that *CsCath D* has 25.25% of alpha helix (100 residues), 31.06% of extended strand (123), 12.37% of beta turn (49) and 31.31% and random coil (124). In addition, I-Tasser program predicted 5 tertiary models

of the protein and based on the C-score value (-0.74), the model 1 was selected for further analysis by Ramachandran plot program. The plot relates that among 396 amino acid residues, 361 residues were presented in favored region (91.6%), 19 residues were in allowed region (4.8%) and only 14 residues were presented in outlier region (3.6%) and confirmed that the predicted model was the proper 3D structure of CsCath D. Further, the structure discloses that the beta sheet formed a 12.37% runs in an antiparallel direction (Fig. 1). A conserved 11 residue active site flap region ends with a beta sheet, where it is predicted to form a cleft for the binding of substrates or inhibitors binding to the active site. The conserved catalytic motif of cathepsin D is usually placed in both N-terminal and C-terminal lobes (prediction from conserved domain database), which is also present in the same region in CsCathD.

Phylogenetic Relationship

The evolutionary tree was constructed using 17 sequences of cathepsin D from birds, fishes, mammals and amphibian. The phylogenetic study admitted that the CsCathD is closely related to the teleost fishes (*L. calcarifer* and *O. latipes*). All the fishes formed one clade which is having 99% of boot strapping after 2000 replicates, when compared with amphibian. Other two clades are mammals and birds which had 100% of boot strapping. Large yellow croaker (90%

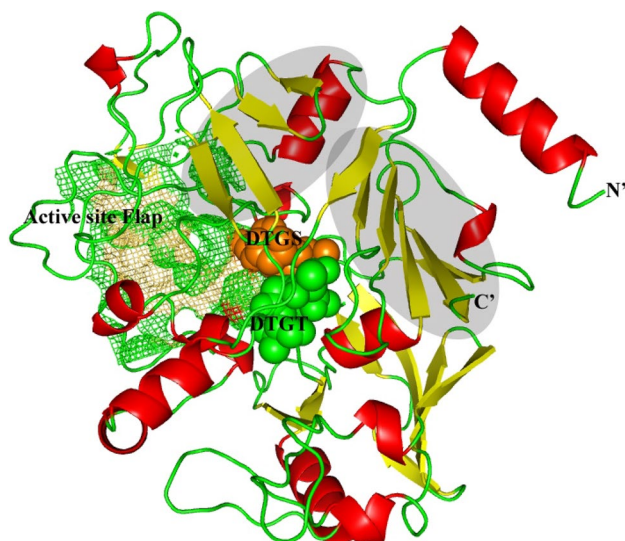


Fig. 1 Three dimensional structure of cathepsin D of *C. striatus*. The 3D structure of CsCath D is presented with red color helix, yellow color beta sheet and green color coils. One of the significant features of cathepsin D is catalytic active sites that are presented at two regions, one at N-terminal region showing orange spheres and other at C-terminal region showing green spheres. An 11 residues active flap site motif which is conserved with amphibian and snake represented as mesh view. Antiparallel β -sheet represented with oval grey background. (Color figure online)

similarities), miiuy croaker and striped beak fish formed an out group from snakehead murrel, however, Asian sea bass and Japanese rice fish is present in the same cluster. The northern white-cheeked gibbon (primate) had more similarities with human, where both are clustered together forming an out group within the same cluster. All birds had a common clade from its ancestor (data shown in Fig. 3 of E-Suppl. File). Overall, the tree topology states the formation of three clades with very less divergence from CsCathD protein sequence.

Antimicrobial Peptide Structure Analysis

The purity of the synthesized peptides was determined by HPLC as 99.17% for PL12 (PLKKFRSIRREL) and 99.13% for NM12 (NITRQAYWQIHM). The antimicrobial peptide in relation to the nature of hydrophobic, cationic and anionic was calculated from PEPTIDE 2.0, an online tool. It was calculated that the N-terminal of PL12 was more hydrophobic (41.67%) and the amount was equal to 41.67% of basic amino acids; in addition, acidic amino acids were equal to the neutral charge obtained (8.33%). The results obtained from 3D model analysis of CsCathD protein revealed the presence of alpha helix at the C-terminal region of peptide P²³-L³⁴ with 5 residues of basic amino acids (Arg and Lys). Helical wheel analysis showed that PL12 is more amphipathic than the other peptide NM12 (data shown in Fig. 4 of E-Suppl. File). Complete prediction proposed that the nature of the peptide to be cationic and hydrophobic. Furthermore, the peptide NM12 at the C-terminal region exposes more hydrophobicity (41.67%) that is equal to the neutral charge (41.67%); and only 16.67% of amino acids were of cationic property and with no anionic property. The overall prediction proposes the nature of peptide to be hydrophobic. Henceforth, both the peptides derived from N-terminal and C-terminal had a putative common feature that goes with all class of antimicrobial peptides property.

MIC Assay

Minimum inhibitory concentration (MIC) of both peptides was determined against various bacterial pathogens. Out of nine fish pathogens used for the study, only three organisms were able to grow less than or equal to one-half of the growth observed in control wells. The MIC of the peptide, PL12 was determined to be 150 μ M against *B. mycooides* and 600 μ M against *S. aureus*. The peptide NM12 (hydrophobic) exhibited MIC at 600 μ M against *K. pneumoniae* and 150 μ M against *B. mycooides* (Fig. 2). Since the inhibitory concentration of PL12 peptide is commendable against *B. mycooides*, further analysis was focused on determination of its mode of action against *B. mycooides*.

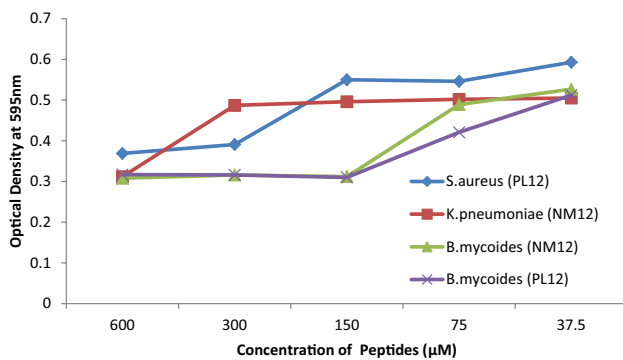


Fig. 2 Minimum inhibitory concentration (MIC) of peptides PL12 and NM12. The X-axis represented the concentration of peptides taken in micro molar concentration and Y-axis represented OD at 595 nm. The inhibitory concentrations of *B. mycooides* for peptides PL12 and NM12 is commendable giving 150 μM which was considered to be the lowest concentration at which observable growth was inhibited

In Vitro Haemolytic Activity

The haemolytic activity of peptide against human RBC was performed to determine the cytotoxicity of peptide PL12. There is no substantial release of hemoglobin in RBC, even at highest concentration of PL12 (600 μM), when compared with PBS treated cells (Data not shown). However, maximum cell lysis and high release of hemoglobin was observed among RBC treated with Triton-X 100 (Positive control). Accordingly, PL12 is indicated with no cytotoxic activity even at higher concentrations.

Cell membrane Disruption by Peptides

The bacterial cells treated with PL12 peptide showed maximum propidium iodide (PI) internalization which is identified by the shift in fluorescence by PI indicated that are denoted by green lines in M2 region. The fluorescence indicates that PI intercalates between base pairs of bacterial DNA after the entry via bacterial membrane lysis. This is in contrast to bacterial cells treated with PBS showing no significant PI internalization that are indicated by red lines in M1 region (Fig. 3). The cell count of nine different bacterial cells with significant PI internalization was determined and the results were tabulated (Table 2). Also, a significant reduction in cell count of *B. mycooides* (37% live cells) was observed in peptide treated cells when compared to that of PBS treated cells (99% live cells) which confirmed the bactericidal potential of the PL12 peptide.

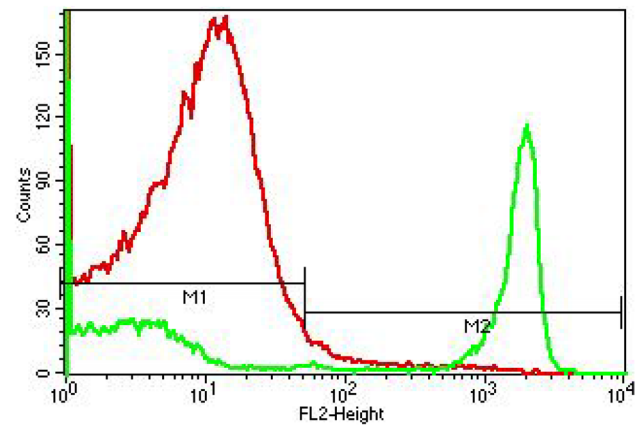


Fig. 3 Cell membrane disruption of Gram positive *Bacilli* by peptide PL12. Internalization of PI by PL12 treated *B. mycooides* cells as assessed by flow cytometry. Bacteria (1×10^6 CFU/ml) were incubated for 180 min with PL12 (green line) showed maximum internalization of PI. Bacteria incubated without peptides showed negligible PI internalization (red line). Numbers indicate the percentage of PI positive bacterial cells after incubation. (Color figure online)

Gene Expression Profiles of CsCathD

The tissue transcript expression analysis of *CsCath D* was conducted using real-time RT-PCR. The transcript was expressed in wide range of tissues without a stimulant and expressed with a distinct pattern of expression in immune related tissues (liver and spleen) and with highest ($P < 0.05$) expression in the head kidney and lowest expression in brain, muscle and skin (Fig. 4a). The data were normalized using beta actin housekeeping gene and about 11 tissue samples of *C. striatus* from various regions were used for the study. Cathepsin D, aspartyl proteases in lysosomes involved in innate immune response. As a first step, to determine the expression of the gene *CsCathD* in response to bacterial, viral residues and fungal infection, we have conducted a time dependent transcription analysis of the *CsCathD* in head kidney after infection with bacteria *A. hydrophila*, poly I:C and fungi *A. invadans*. The assay was performed using head kidney because, this tissue constitutively expressed more number of *CsCath D* transcripts. The analysis of cathepsin D expression in response to *A. hydrophila* showed a significant ($P < 0.05$) up regulation in head kidney at 24 h post-infection (p.i) and was followed by significant down regulation at 48 h p.i and reached near to basal level of expression at 72 h p.i (Fig. 4b). The same pattern was observed with poly I:C infected fish (Fig. 4c). Following challenge with *A. invadans*, the transcript level was substantially increased ($P < 0.05$) at 48 h starting from 12 h p.i., thereby reduces at 72 h p.i. (Fig. 4d). The fold change was calculated relative to the corresponding time of PBS injection control. During the challenge no mortality of fish was observed.

Fig. 4 Gene expression pattern of *CsCathD*. **a** Basal expression pattern of *CsCathD* in different tissues of *C. striatus*; **b** pathogen-induced expression pattern of *CsCathD* during bacterial infection; **c** poly I:C induced modulation of expression pattern of *CsCathD*; **d** post-fungal induced expression modulation of *CsCathD*

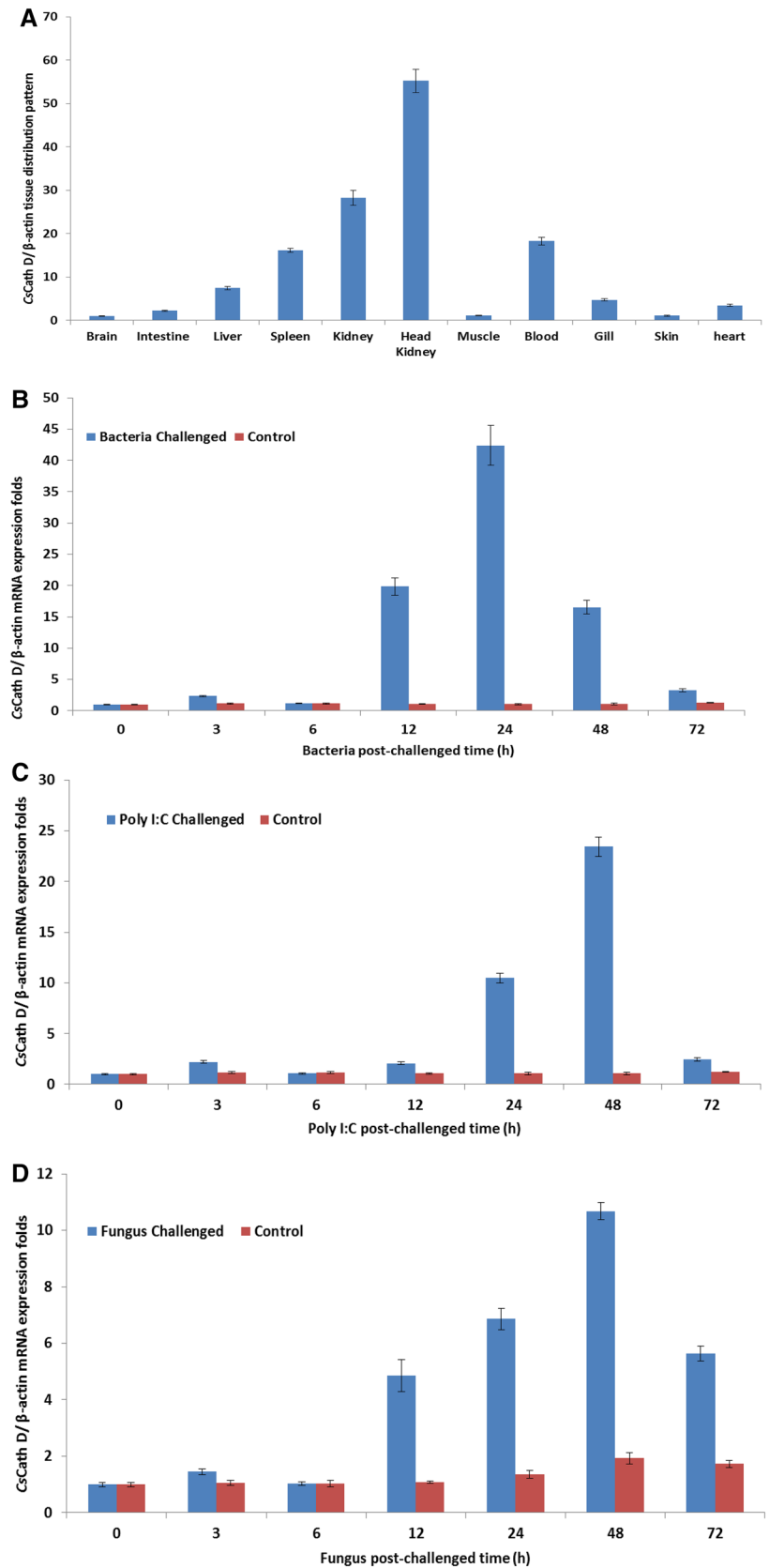


Table 2 Propidium iodide internalization in nine different bacterial cells post-treatment with 600 μ M of NM12 and PL12 peptide analyzed by flow cytometer method

S. no.	Bacteria	Events/15 s	PI stained cells	% of PI stained cells
PL12				
1	<i>Bacillus mycoides</i>	30,997	22,999	74.2
2	<i>Bacillus cereus</i>	72,499	21,387	29.5
3	<i>Bacillus subtilis</i>	55,402	18,615	33.6
4	<i>Escherichia coli</i> (ATCC 25922)	38,574	18,708	48.5
5	<i>Escherichia coli</i> (ATCC 9637)	61,121	26,221	42.9
6	<i>Klebsiella pneumoniae</i>	34,002	21,931	64.5
7	<i>Staphylococcus aureus</i>	46,682	17,926	38.4
8	<i>Pseudomonas aeruginosa</i>	51,421	20,517	39.9
9	<i>Micrococcus luteus</i>	52,662	23,382	44.4
NM12				
1	<i>Bacillus mycoides</i>	44,254	8895	20.1
2	<i>Bacillus cereus</i>	75,531	11,707	15.5
3	<i>Bacillus subtilis</i>	59,421	7844	13.2
4	<i>Escherichia coli</i> (ATCC 25922)	66,532	12,974	19.5
5	<i>Escherichia coli</i> (ATCC 9637)	63,135	16,036	25.4
6	<i>Klebsiella pneumoniae</i>	58,525	26,826	45.8
7	<i>Staphylococcus aureus</i>	51,339	30,136	58.7
8	<i>Pseudomonas aeruginosa</i>	53,109	8179	15.4
9	<i>Micrococcus luteus</i>	55,588	11,562	20.8

Discussion

The abrupt emergence of bacterial infections that are resistance to many drugs underscores the need for new therapeutic agents (Pinner 1996; Koplan 1998; Khurshid 1999). The cationic antimicrobial peptides (CAP) are synthesized by all organisms including animals, plants and aquatic organisms; they are recognized by the major part of the defense system of organisms (Hancock and Scott 2000). In addition, the categorized antimicrobial peptides (AMPs) to be a basic proteins and polypeptides that combine with cell nucleoproteins or other negatively charged surface constituents of bacteria; and were being inducible on exposure to infecting microorganisms that aids allied mechanisms of natural and adaptive immunity (Kim and Brogden 2005). The present study focuses on the development of CAP generated from complete protein, which are gene encoded peptides that can kill bacteria by disrupting their cell membrane.

As a first step in the current study, we have obtained computational approaches for understanding the molecular basis of gene cathepsin D of *C. striatus*. The complete sequence of cathepsin D of *C. striatus* has been obtained from an already developed cDNA library (Kumaresan et al. 2014). CsCathD had maximum identity of 90% with large yellow croaker (fish), also had similarities with chicken, birds, amphibians and mammals; whereas the lowest identity of 69% was observed with pig-tailed macaque monkey. This

is in contrast to the studies of grass carp cathepsin D gene by Dong et al. (2012) who showed the highest amino acid identity of 91% to *Danio rerio*, followed by *Ictalurus punctatus* (88%) and had lowest identity of 48% to *Meloidogyne incognita*. The fundamental relatedness of CsCathD with other species of cathepsin D from phylogenetic analysis reveals the formation of three clades consisting of mammals, birds followed by fishes. The data obtained from the phylogenetic analysis of CsCathD displays all cathepsin D from teleost fishes clustered into one group at first and then the amphibians, mammals and poultry as formed a larger vertebrate group; the invertebrates were in a different cluster.

In general most of the trans-membrane proteins have signal peptides, capable of initiating signals that are responsive to the external environment of the cell. Accordingly, in CsCath D, there is a signal peptide region at 1–17 of N terminal domain and the signal cleavage site between Ala¹⁷ and Ala¹⁸ indicating the role of signal peptidase, which correlates with the studies of pre-pro-cathepsin D from grass carp showing the signal peptide cleavage site between Ala¹⁸ and Ile¹⁹ at the N-terminal region (Gao et al. 2012). This pre-pro-cathepsin D after cleavage of the signal peptide region is converted into pro-cathepsin D and undergoes several cleavage to produce the mature protein upon entering the lysosome. In addition, the catalytic motif DTGT and DTGS in both N terminal and C terminal lobes are strictly conserved and each motif contributes catalytic residues D⁹⁴ and D²⁸¹

which is aspartic acid (D), characteristic of a pepsin family of proteinases showing the protein to be aspartic protease. It is involved in the proteolytic activity of the target molecule being attached to the active site flap region presented as a cleft separated between the walls of two lobes. The protein is presented with active flap site of 11 residues forming an extended loop projecting as a cleft for the binding of substrates or inhibitors within the active site. It is observed from the multiple sequence analysis that the catalytic residues and the catalytic active sites (DTGT) and (DTGS) are conserved with fish, snake, frog and human. Nevertheless the multiple sequence analysis of cathD from *Paralichthys olivaceus* (Park et al. 2009) revealed only the presence of one catalytic site (DTG) of the deduced protein which is well conserved. By definition, the cathepsin D exists as single-chain or two chain enzyme. Shewale and Tang (1984) states that an insertional sequence specific for cathepsin D involved in the proteolytic processing of the single-chain to two-chain form and observed in vivo studies. However, the current multiple sequence alignment of CsCathD indicates the presence of insertional sequence “PCSASSASAL” only for humans but not for fish, chicken, snake and frog. Mori et al. (1997) also reported that the insertion is lacked in olive flounder cathepsin, suggesting that it exists as single-chain enzyme, like other aspartic proteases. Similarly, cathepsin D of *C. striatus* is also a single chain enzyme, which is lacking the insertion sequence. The structural features of CsCathD relates the presence of alpha helix and beta pleated sheets in antiparallel direction indicating the characteristic feature of beta sheet peptides, derived from this protein.

The studies on phylogenetic relationship of CsCathD against all other species articulate overall topology showing the formation of three clades with only very less divergence from CsCathD protein sequence. In contrast, the understandings of Jia and Zhang (2009) obtained a phylogenetic based on the amino-acid sequences of turbot cathepsin D and other aspartic proteinases in different organisms stating, turbot cathepsin D to be most similar to cathepsin D from Barramundi or Asian seabass (*L. calcarifer*), and also had high similarity with those from other organisms, especially from fish. Further they (Jia and Zhang 2009) stated that the other groups of aspartic proteinases formed distinct paraphyletic clusters, respectively. Similarly the CsCathD is closely related to *L. calcarifer* and other fishes group. The studies on turbot cathepsin D denote the evolutionary relationship and functional similarities of CsCathD.

Preliminary sequence analysis of CsCathD showed that they have equal number of positive and negatively charged residues which makes the protein amphipathic in nature. This urged us to focus on the regions with putative antimicrobial regions. Based on the antimicrobial propensity value of amino acids, two regions of CsCath D have been identified as antimicrobial region and they were designed

and named in accordance with previous reports. Peptides, one from N-terminal PL12 hold an alpha helix and other from C-terminal NM12 with beta sheet, signifying the possession of certain antimicrobial peptide property. In general, antimicrobial peptides have a common feature of having positive charge, hydrophobic or amphipathic. The results obtained from peptide calculator suggests that the peptide PL12 to be cationic and hydrophobic having approximately 45% of arginine and lysine residues; and peptide NM12 to be more hydrophobic against cationic nature. Also, helical wheel analysis showed that PL12 is more amphipathic than the other peptide NM12. Generally amphipathic peptides are more active on bacterial membranes as they comprise both hydrophilic and hydrophobic faces. Therefore, both the peptides exhibited all the properties that are required for an antimicrobial peptide.

Antimicrobial assays showed that both the peptides showed greater affinity towards Gram positive Bacilli. Although MIC of 300 μM for PL12 had activity towards Gram positive cocci and 150 μM for Gram positive Bacilli, there is sudden increase in the growth of microbial cells thereafter. Similarly, peptide NM12 had greater affinity towards Gram positive Bacilli. Generally from the previous studies cationic anti-microbial peptides inhibits the growth of Gram positive bacteria, Gram negative bacteria, fungi and enveloped viruses. In this study, we have derived the CAP and showed the activity against bacteria only. The present work was focused further to study the property of CAP, since it showed inhibitory effect against *B. mycooides*, which was commendable. However at higher concentrations, both the peptides exhibited inhibitory activity against *K. pneumonia* and *S. aureus* which explain the significance of the biological activity of both the peptides (Table 2). Propidium iodide internalization was observed in most of the tested bacteria which emphasize the action of those peptides on the membrane of the tested bacteria. Overall, PL12 was identified with better activity at comparatively lower concentration than NM12 and therefore the mechanism of action of PL12 was determined against *B. mycooides* to understand the mode of action of the peptide on the bacterial cell.

Cationic antimicrobial peptides are generally made of alpha helices and beta sheets, accordingly the peptides derived from cathepsin D of *C. striatus* was found to be having alpha helix and beta sheet (Hancock and Lehrer 1998). The hypothesis put forth by Piers et al. (1993) relates the activity of peptides cationic nature towards the negatively charged cell membrane of bacteria which allowed distortion by neutralizing charge over the cell membrane of bacteria. In agreement with the previous studies, the cationic and hydrophobic nature of PL12 could able to lyse the cell membrane of *B. mycooides*, which was confirmed by dye exclusion assay using propidium iodide (PI) internalization and observed in flow cytometer. The peptide treated bacterial cells was

detected by fluorescence produced by PI which intercalates between base pairs of bacterial DNA after entering via cell membrane cleavage. A similar kind of membrane disruption ability was exhibited by a fish lysozyme derived antimicrobial peptide which also comprised 12 amino acids (Kumaresan et al. 2015a, b). Although several reviewers have suggested cell membrane cleavage as an outcome, there is little evidence for complete destruction of bacterial cells. But to some extent we say that the interaction of the peptide upon treatment with bacteria has caused distortion showing its vital role in the bacterial cell membrane lysis. Cytotoxicity assay revealed that the peptide remained non-toxic against human RBC. This supports that PL12, a derived antimicrobial peptide from *CsCathD* to be an intoxicating agent presumed to be used as an alternative for antibiotics and could also be subjected as adjuvants during vaccine development for initial recognition of fish pathogen where several species of fish being reared at high densities in the aquaculture culture system.

To understand better about the immune related functions of cathepsin D, it is of prime importance to analyze the cathepsin D activity further, and the expression kinetics and localization of cathepsin D in response to infection which was conducted by RT PCR. The expression profile of *CsCathD* showed abundant relative transcript expression in the head kidney and lowest expression in brain, muscle and skin. This divergence of expression patterns is in agreement of duplicated genes that can often tend to share functional partitioning through tissue expression differences (Cresko et al. 2003). The gene transcripts of epinecidin-1, a well-known antimicrobial peptide from *Epinephelus coioides* were most abundantly expressed in the head kidney and intestine (Pan et al. 2007). While comparing with other reports, our results supported the hypothesis that *CsCathD* expression occurs in the head kidney for defense against pathogens and may play an important role in the first line of defense mechanism as nonspecific innate immunity. The present efforts of gene expression technique comprise a time dependent transcription analysis of the *C. striatus* cathepsin D in head kidney after infection with bacteria *A. hydrophila*, poly I:C and fungus *A. invadans*. The analysis of *CsCathD* expression in response to *A. hydrophila* showed a substantial up regulation in head kidney at 24 h p.i and up regulation was followed by considerable down regulation at 48 and 72 h p.i. In contrast to expression of the catfish cathepsin D (Feng et al. 2011) showed varied changes in the tested tissues following challenge. And showed its significant transcript up regulation in head kidney at 4 h and in liver at 24 h. Upregulation in head kidney was followed by significant down-regulation at 24 h and 72 h. The result of catfish cathepsin D was disparate to the outcomes of *CsCathD* in

agreement with substantial up regulation and down regulation. Whereas, the expression level of cathepsin D was constitutive and increased greatly during post-infection revealed that this protein might protect against bacterial infection. Similarly, poly I:C and challenge with *A. invadans* had substantially increased at 48 h starting from 12 h p.i, thereby reduced at 72 h p.i. These results demonstrated the role of *CsCathD* in eliminating the pathogens. Although it is not clear why *CsCathD* mRNA expression was upregulated at 24 h for bacteria and 48 h for poly I:C and fungus and then down regulation during post-infection, we speculate that cathepsin D plays a potent role in host defense against infectious diseases, and is also an important component of innate immunity.

The gene expression modulation of *CsCathD* during bacterial and fungal infections and the antimicrobial role of NM12 against *B. mycoides* together suggest the inevitable role of *CsCathD* as an antimicrobial agent and the identified peptide could be developed as a therapeutic agent to treat *B. mycoides* infection; however, further clinical trials are required to develop it as a successful pharmaceutical candidate.

Acknowledgements This research is supported by Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India, New Delhi through DBT's Prestigious Ramalingaswami Re-entry Fellowship (BT/RLF/Re-entry/27/2011). The corresponding author is grateful to the Deanship of Scientific Research, King Saud University for partial funding through Vice Deanship of Scientific Research Chairs. Also, the corresponding author would like to acknowledge Universiti Putra Malaysia, Malaysia for providing him visiting professor award (UPM/PEND/500-3/4/10) to complete this study under the HICoE Program, Ministry of Higher Education Malaysia.

Funding This study was funded by Department of Biotechnology (BT/RLF/Re-entry/27/2011), Ministry of Science and Technology, Government of India, New Delhi; Deanship of Scientific Research, King Saud University and Universiti Putra Malaysia (UPM/PEND/500-3/4/10).

Compliance with Ethical Standards

Conflict of interest Akila Sathyamoorthi, Venkatesh Kumaresan, Rajesh Palanisamy, Mukesh Pasupuleti, Mariadhas Valan Arasu, Naif Abdullah Al-Dhabi, Kasi Marimuthu, S. M. Nurul Amin, Aziz Arshad, Fatimah Md. Yusoff and Jesu Arockiaraj declare that they have no conflict of interest.

Ethical Approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional [SRM University (361/IEC/2012) and CSIR-CDRI, Lucknow (CDRI/IEC/2014/A1)] and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

References

- Arasu A, Kumaresan V, Sathyamoorthi A, Arasu MV, Al-Dhabi NA, Arockiaraj J (2016) Coagulation profile, gene expression and bioinformatics characterization of coagulation factor X of striped murrel *Channa striatus*. *Fish shellfish immunol* 55:149–158
- Arockiaraj J, Haniffa MA, Seetharaman S, Perumalsamy PRR (2004) Utilization of lipid as dietary energy source for fingerlings of *Channa striatus*. *Malays J Sci* 23(2):1–5
- Arockiaraj J, Bhatt P, Kumaresan V, Dhayanithi NB, Arshad A, Hari Krishnan R, Al-Dhabi NA (2015) Fish chemokines 14, 20 and 25: a comparative statement on computational analysis and mRNA regulation upon pathogenic infection. *Fish shellfish immunol* 47(1):221–230
- Baechle D, Flad T, Cansier A, Steffen H, Schittek B, Tolson J, Herrmann T, Dihazi H, Beck A, Mueller GA, Mueller M, Stevanovic S, Garbe C, Mueller CA, Kalbacher H (2006) Cathepsin D is present in human eccrine sweat and involved in the postsecretory processing of the antimicrobial peptide DCD-1L. *J Biol Chem* 281(9):5406–5415
- Baldocchi RA, Tan L, King DS, Nicoll CS (1993) Mass spectrometric analysis of the fragments produced by cleavage and reduction of rat prolactin: evidence that the cleaving enzyme is cathepsin D. *Endocr* 133(2):935–938
- Baricos WH, Zhou YW, Fuerst RS, Barrett AJ, Shah SV (1987) The role of aspartic and cysteine proteinases in albumin degradation by rat kidney cortical lysosomes. *Arch Biochem Biophys* 256(2):687–691
- Boman HG (1995) Peptide antibiotics and their role in innate immunity. *Annu Rev Immunol* 13:61–92
- Cho JH, Park IY, Kim HS, Lee WT, Kim MS, Kim SC (2002a) Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. *J FASEB* 16(3):429–431
- Cho JH, Park IY, Kim HS, Lee WT, Kim MS, Kim SC (2002b) Cathepsin D produces antimicrobial peptide parasin I from histone H2A in the skin mucosa of fish. *FASEB J*. 16(3):429–431
- Cole AM, Weis P, Diamond G (1997) Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. *J Biol Chem* 272(18):12008–12013
- Cresko WA, Yan YL, Baltrus DA, Amores A, Singer A, Rodriguez-Mari A (2003) Genome duplication, subfunction partitioning, and lineage divergence: Sox9 in stickleback and zebrafish. *Dev Dyn* 228(3):480–489
- Dong ZD, Zhang J, Ji XS, Zhou FN, Fu Y, Chen WY, Zeng YQ, Li TM, Wang H (2012) Molecular cloning, characterization and expression of cathepsin D from grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol* 33:1207–1214
- Ellis AE (1982) Differences between the immune mechanisms of fish and higher vertebrates. In: Robert R (ed) *Microbial disease of fish*. Academic Press, London, pp 1.30
- Feng T, Zhang H, Liu H, Zhou Z, Niu D, Wong L, Kucuktas H, Liu X, Peatman E, Liu Z (2011) Molecular characterization and expression analysis of the channel catfish cathepsin D genes. *Fish Shellfish Immunol* 31:164–169
- Gao L, He C, Liu X, Su H, Gao X, Li Y, Liu W (2012) The innate immune-related genes in catfish. *Int J Mol Sci* 13(11):14172–14202
- Hancock REW, Lehrer RI (1998) Cationic peptides: a new source of antibiotics. *Trends Biotechnol* 16:82–88
- Hancock REW, Scott MG (2000) The role of antimicrobial peptides in animal defences. *Proc Natl Acad Sci USA* 97:8856–8861
- Jayaram KC (1981) *Freshwater fishes of India, Pakistan, Bangladesh, Burma and Srilanka. A handbook, 1st edn.* Zoological Survey of India, Calcutta
- Jia A, Zhang XH (2009) Molecular cloning, characterization and expression analysis of cathepsin D gene from turbot *Scophthalmus maximus*. *Fish Shellfish Immunol* 26:606–613
- Khurshid MA (1999) *Staphylococcus aureus* with reduced susceptibility to vancomycin. *Morbidity Mortality Weekly Rep* 48:1165–1167
- Kim A, Brogden (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria. *Nat Rev Microbiol* 3(3):238–250
- Koplan JP (1998) Preventing emerging infectious diseases. A strategy for the 21st century. US Department of Health and Human Services, Centers for Disease Control and Prevention, Atlanta
- Kumaresan V, Bhatt P, Palanisamy R, Gnanam A, Pasupuleti M, Arockiaraj J (2014) A murrel cysteine protease, cathepsin L: bioinformatics characterization, gene expression and proteolytic activity. *Biologia* 69(3):395–406
- Kumaresan V, Gnanam AJ, Pasupuleti M, Arasu MV, Al-Dhabi NA, Hari Krishnan R, Arockiaraj J (2015a) Comparative analysis of CsCu/ZnSOD defense role by molecular characterization: gene expression-enzyme activity-protein level. *Gene* 564(1):53–62
- Kumaresan V, Bhatt P, Ganesh MR, Hari Krishnan R, Arasu M, Al-Dhabi NA, Pasupuleti M, Marimuthu K, Arockiaraj J (2015b) A novel antimicrobial peptide derived from fish goose type lysozyme disrupts the membrane of *Salmonella enterica*. *Mol Immunol* 68(2):421–433
- Kumaresan V, Ravichandran G, Nizam F, Dhayanithi NB, Arasu MV, Al-Dhabi NA, Arockiaraj J (2016) Multifunctional murrel caspase 1, 2, 3, 8 and 9: conservation, uniqueness and their pathogen-induced expression pattern. *Fish shellfish immunol* 49:493–504
- Lapresle C, Puizdar V, Porchon-Bertolotto C, Joukoff E, Turk V (1986) Structural differences between rabbit cathepsin E and cathepsin D. *Biol Chem Hoppe Seyler* 367:523–526
- Lauth X, Shike H, Burns JC, Westerman ME, Ostland VE, Carlberg JM, Van Olst JC, Nizet V, Taylor SW, Shimizu C, Bulet P (2002) Discovery and characterization of two isoforms of moronecidin, a novel antimicrobial peptide from hybrid striped bass. *J Biol Chem* 277:5030–5039
- Lee DC, Womble TA, Mason CW, Jackson IM, Lamango NS, Severs WB (2007) Hydroxydopamine induces cystatin C-mediated cysteine protease suppression and cathepsin D activation. *Neurochem Int* 50:607–618
- Lilley JH, Callinan RB, Chinabut S, Kanchanakhan S, MacRae IH, Phillips MJ (1998) Epizootic ulcerative syndrome (EUS) technical handbook. The Aquatic Animal Health Research Institute, Bangkok, p 88
- Magnadottir B (2006) Innate immunity of fish (overview). *Fish Shell Immunol* 20(2):137–151
- Mori K, Ogawa Y, Tamura N, Ebihara K, Aoki T, Muro S, Ozaki S, Tanaka I, Tashiro K, Nakao K (1997) *FEBS Lett* 20:218–222
- Palanisamy R, Bhatt P, Kumaresan V, Pasupuleti M, Arockiaraj J (2016) Innate and adaptive immune molecules of striped murrel *Channa striatus*. *Rev Aquacult*. <https://doi.org/10.1111/raq.12161>
- Pan CY, Chen JY, Cheng YS, Chen CY, Ni IH, Sheen JF, Pan YL, Kuo CM (2007) Gene expression and localization of the epinecidin-1 antimicrobial peptide in the grouper (*Epinephelus coioides*), and its role in protecting fish against pathogenic infection. *DNA Cell Biol* 26(6):403–413
- Park CB, Lee JH, Park IY, Kim MS, Kim SC (1997) A novel antimicrobial peptide from the loach, *Misgurnus anguillicaudatus*. *FEBS Lett* 411:173–178
- Park IY, Park CB, Kim MS, Kim SC (1998) Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*. *FEBS Lett* 437:258–262
- Park EM, Kim Y, Nam BH, Hong HJ, Kim WJ, Lee SJ, Kim KK (2009) Cloning and expression analysis of cathepsin D in the olive flounder *Paralichthys olivaceus*. *Biosci Biotechnol Biochem* 73(8):1856–1859

- Patrzykat A, Zhang L, Mendoza V, Iwama GK, Hancock REW (2001) Synergy of histone-derived peptides of coho salmon with lysozyme and flounder pleurocidin. *Antimicrob Agents Chemother* 45(5):1337–1342
- Piers KL, Brown MH, Hancock RE (1993) Recombinant DNA procedures for producing small antimicrobial cationic peptides in bacteria. *Gene* 134(1):7–13
- Pinner RW (1996) Trends in infectious diseases mortality in the United States. *J Am Med Assoc* 275:189–193
- Ravichandran G, Kumaresana K, Valan Arasuc M, Al-Dhabi NA, Ganesh MR, Mahesh A, Dhayalane A, Pasupuleti M, Arockiaraj J (2016) Pellino-1 derived cationic antimicrobial prawn peptide: bactericidal activity, toxicity and mode of action. *Mol Immunol* 78:171–182
- Shewale JG, Tang J (1984) Amino acid sequence of porcine spleen cathepsin D. *Proc Natl Acad Sci USA* 81(12):3703–3707
- Shike H, Lauth X, Westerman ME, Ostland VE, Carlberg JM, Van Olst JC, Shimizu C, Bulet P, Burns JC (2002) Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge. *Eur J Biochem* 269:2232–2237
- Tang J, Wong RN (1987) Evolution in the structure and function of aspartic proteases. *J Cell Biochem* 33:53–63
- Wu M, Hancock RE (1999) Improved derivatives of bactenecin, a cyclic dodecameric antimicrobial cationic peptide. *Antimicrob Agents Chemother* 43:1274–1276