E-ISSN: 2347-5129
P-ISSN: 2394-0506
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.549
IJFAS 2020; 8(4): 124-129

www.fisheriesjournal.com
Received: 18-05-2020
Accepted: 22-06-2020

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Cloning and sequence analysis of Astacin like metalloproteinase gene from Indian oyster, *Magallana bilineata* (Röding, 1798)

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Abstract
Astacins are a group of zinc-dependent metalloproteases with diverse roles in biological systems such as embryonic development, connective tissue remodelling, digestion, and processing of biologically active peptides. This paper describes the cloning of an Astacin like metalloproteinase (*Mbi-ALMP*) from the digestive tissues of *Magallana bilineata*. The *Mbi-ALMP* encodes a deduced 440 amino acid protein which includes an N-terminal signal peptide. The *Mbi-ALMP* possesses several of the characteristic features of the Astacin family like the pro-peptide domain, Astacin catalytic domain with Zinc binding motif, and met-turn, followed by a MAM (meprin, A5 protein, receptor protein-tyrosine phosphatase μ) carboxy-terminal domain. Amino acid sequence alignment and phylogenetic analysis of the protease domain indicate *Mbi-ALMP* to have a high degree of sequence homology to other Astacin like metalloproteases from different species.

Keywords: *Magallana bilineata*, Astacin, MAM, metalloprotease, *Mbi-ALMP*

1. Introduction
Astacins are a family of multidomain zinc-dependent metalloendopeptidase that have diverse roles in biological systems, ranging from connective tissue remodelling to processing of biologically active peptides [1] The family is named after the digestive protease "Astacin" -first reported by Zwilling and colleagues in the 1960s, from the digestive tract of the freshwater crayfish *Astacus astacus* L. [2].This purely digestive protein has been intensively studied with respect to its structure [3], enzymatic properties [4], and genetic organization [5]. It is also the prototype for the Astacin family of extracellular zinc endopeptidases, members of which are found across the animal kingdom including vertebrates and invertebrates as well as in some bacterial species.

The basic structure of Astacins comprises of several domain motifs: -N terminal signal and pro-sequences, a catalytic domain, and multiple C terminal domains [6]. The characteristic feature of the Astacin family is its catalytic domain which has a highly conserved region containing the consensus sequences "HEXXHXXGFXHEXX" with the Zinc binding motif "HEXXH" and a conserved methionine containing turn (Met-turn) "SXMHY". The approximately 200 amino acid residue catalytic domain of Astacins has strikingly similar 3-dimensional structure despite low amino acid sequence identity [7, 1]. The mature active crayfish astacin has a minimal domain structure with just the catalytic domain. However, most astacins are multidomain proteins consisting of complex carboxy-terminal elongations with one or more regulatory or interactive domains in addition to the catalytic domain [8].

All members of the Astacin family can be roughly classified based on their functions and carboxy-terminal elongations into three categories *viz* Meprins, Bone Morphogenetic Protein (BMP-1)/tolloids and Hatching related enzymes. Meprins are membrane-bound enzymes expressed mostly in the highly differentiated epithelium tissues playing crucial roles in the processing of biologically active peptides and extracellular matrix proteins [9, 8]. The second class of proteins BMP-1/tolloids includes morphogenetically active proteins found in vertebrates and invertebrates contributing to morphogenesis or embryogenic pattern formation [10]. The third class of Astacins are involved in the breaking of egg envelopes during the hatching of Xenopus [11], fish [12, 13], and birds [14].

To date, several hundreds of Astacins have been reported in animals and bacteria [1] and the number is rapidly increasing due to several ongoing genome sequencing projects. No Astacins have yet been identified in plants or viruses [15]. According to the MEROPS, the database for peptidases, the genome of lower vertebrates and invertebrates has more Astacin genes than mammalian genomes: 7 to 18 in amphibians and fish, at least 4 in cnidarians, 13 to 25 in insects, and up to 40 in nematodes such as *Caenorhabditis elegans* [16].

In this paper, we report the identification and cloning of *Mbi-ALMP*, an Astacin like metalloproteinase from the gut tissue of the Indian Oyster *Magallana bilineata*. Currently, there are only few reports of Astacin metalloendopeptidases from bivalves and most of the data available are based on predictions from computational analysis. An Astacin like metalloproteinase with suggested roles in oyster immunity and development processes was identified from the pearl oyster, *Pinctada fucata* [6]. Similar Tolloid like gene/ BMP-1 was also identified from *Crassostrea ariakensis* upon stimulation with Rickettsia like organisms (RLO) [17]. Biomineralization of molluscan shells and Molluscan immunity has long been the favoured subjects of study. Bivalves such as oysters are considered as model organisms to study developmental biology due to their mosaic development; the impact of ocean acidification on marine organisms as well as molluscan immunity due to their resilient nature [18, 19]. Because of the importance of Astacins in embryo development, morphogenesis, extracellular matrix deposition, digestion, and immunity, identifying and characterizing Astacins from bivalves will contribute to understand the relative function of these enzymes in molluscs.

2. Materials and Methods

2.1 Animal Collection and Maintenance

Adult specimens of *M. bilineata* were sampled from Sathar Island region of Kodungallur-Azhikode estuary (10°11′26.34″N and 76°11′28.88″E), Ernakulam, India. The sampled oysters were transferred to wet laboratory (CMFRI hatchery) and maintained in filtered and aerated water of salinity 22 ppt & water temperature 25-28ºC. The animals were held as described for a period of 7 days to acclimate to the laboratory conditions. The digestive tissue was excised from the oysters and immediately transferred to RNAlater stabilization solution (Thermo Fisher Scientific, USA) and frozen at -80ºC.

2.2 Total RNA Isolation

Total RNA was extracted using Trizol reagent (Ambion life technologies, USA) from the digestive tissue, as per manufacturer's protocol. Samples were then treated with DNAse I (Sigma) (1 U/µg total RNA). The integrity and quality of total RNA was checked using a spectrophotometer, Biophotometer plus (Eppendorf, Germany), at 260 nm and further in 1.5% agarose gel electrophoresis. The RNA isolated was used for cDNA synthesis and RACE PCR.

2.3 Full-length cDNA cloning of Mbi-ALMP

The partial sequence of *Mbi-ALMP* (MH887439) was obtained from a previous study which involved the construction of a Suppression Subtractive Hybridization cDNA library from the digestive tissues of *M. bilineata*. In order to generate the complete reading frame of *Mbi-ALMP*, RACE PCR was carried out using SMART RACE cDNA amplification kit (Clonetech, USA) in both the 5' and 3' directions. Both the RACE primers (*Mbi-ALMP* 3' RACE primer: 5'CCACTTCCAGAAGACGGCCAAGTTAGA3' and *Mbi-ALMP* 5' RACE primer: 5'GGTAACCCCTACCGGTATGTCCCTCAA3') were designed using primer3 plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and synthesized by Vision Scientific, India.

A total of 1 µg of RNA was used as template for the RACE PCR. 3' and 5' reactions were performed using klentaq HF polymerase with a gene-specific primer paired with a RACE universal primer, in a step-down protocol. Both the 3' and 5' products generated were ligated and cloned onto the pJET1.2 cloning vector system. The clones were then sequenced in both directions and contig aligned with the help of Seqman (Lasergene) assembly program. The aligned contigs were analysed using NCBI blast for comparison with known cDNAs as well as identification of orthologs.

2.4 Sequence Analysis

The open reading frame (ORF) and the amino acid sequence of *Mbi-ALMP* gene were determined using EditSeq which is a sub-module of Lasergene software. The molecular weight and the pI value of *Mbi-ALMP* were predicted using the same software. The motif sequences were predicted with the aid of InterPro Scan software (http://www.ebi.ac.uk/InterProScan/) and PROSITE program (http://kr.expasy.org/prosite/). The software SignalP 4.1 (www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide sequences. A multiple sequence alignment was generated using homologous Astacin like metalloendopeptidase gene sequences from other species obtained from NCBI blast query, using Bio-Edit multiple alignment tool and clustal w [20]. Finally, a phylogenetic tree was constructed with MEGA software version 7.0.21 using the neighbour joining method [21].

2.5 Ethical Statement

All experimental protocols were approved and regularly assessed by the Institute Research Council (IRC) of ICAR-Central Marine Fisheries Research Institute. The Priority Setting, Monitoring, and Evaluation (PME) cell of ICAR-Central Marine Fisheries Research Institute have approved the manuscript for submission considering set criteria, including ethical aspects

3. Results and Discussion

Astacins are Zinc dependent multifunctional metalloproteinases that have various physiological functions [6, 22]. The members of the Astacin family are known to have manifold functions in metabolism such as processing of biologically active peptides and matrix proteins, extracellular matrix assembly, digestion, and cleavage of egg envelopes during hatching [1, 23]. Homologous sequences from this highly diverged protein family can be found in both vertebrate and invertebrate species. However, studies conducted on Astacin like metalloendopeptidase in molluscs, especially bivalves, are few. Astacin like proteins were found to be up-regulated upon bacterial infection in *Crassostrea gigas* and lipo-polysaccharide (LPS) challenge in *P. fucata*. A bone morphogenic protein (BMP-1), a matrix metalloproteinase and an ADAM ("a metalloprotease and disintegrin") protein have also been previously reported in *Crassostrea sp.* [24, 6, 17].

Most of the identified proteins have not been fully characterised and their relative function in the animals are unknown. Therefore, characterization of new Astacin family members in bivalves will improve our understanding of how these proteins function and also help in determining its different biological roles in the organism.

In this study, we successfully isolated and sequenced a full-length Astacin like metalloendopeptidase (*Mbi-ALMP*) cDNA from the digestive tissues of *M. bilineata*. The total length of *Mbi-ALMP* is 1431bp with an untranslated region of 42 bp in the 5' region and a 3' untranslated region of 69 bp. This sequence has been deposited in GenBank under Accession No:"MN889547". The ORF of the protein was found to be 1320 bp in length. The deduced amino acid sequence of the coding *Mbi-ALMP* consists of 440 residues with a calculated molecular weight of 49 KDa. The full-length sequence of the cDNA and deduced amino acids of the astacin gene are given in figure 1. The predicted pI value of the putative gene was 6.580 with 33 strongly basic (+) amino acids (K, R), 43 strongly acidic (−) amino acids (D, E), 120 hydrophobic amino acids (A, I, L, F, W, V) and 138 polar amino acids (N, C, Q, S, T, Y). A blast p analysis conducted showed 100% similarity to Astacin like protein of *C. gigas* (XP_011415612.1), 54.21% similarity with *Mizuhopecten yessoensis* (XP_021341865.1) and 38.54% with *Sepiella maindroni* (AGF33810).

The open reading frame of *Mbi-ALMP* indicates that this gene encodes:-(i) a signal peptide (ii) a pro-peptide which is proteolytically cleaved to generate the functional mature protein (iii) a conserved zinc-metalloendopeptidase catalytic domain and a (iv) MAM (meprin, A5 protein, receptor protein-tyrosine phosphatase μ) domain. Astacin family of proteins can occur either as membrane-bound or secreted enzymes. Generally, Astacins are secreted proteases which require the removal of the signal peptide and pro-peptide domain for optimal activity [7, 25, 17]. Both the mentioned features can be observed in *Mbi-ALMP* implying that the enzyme exerts its function in the extracellular space as a secreted protein.

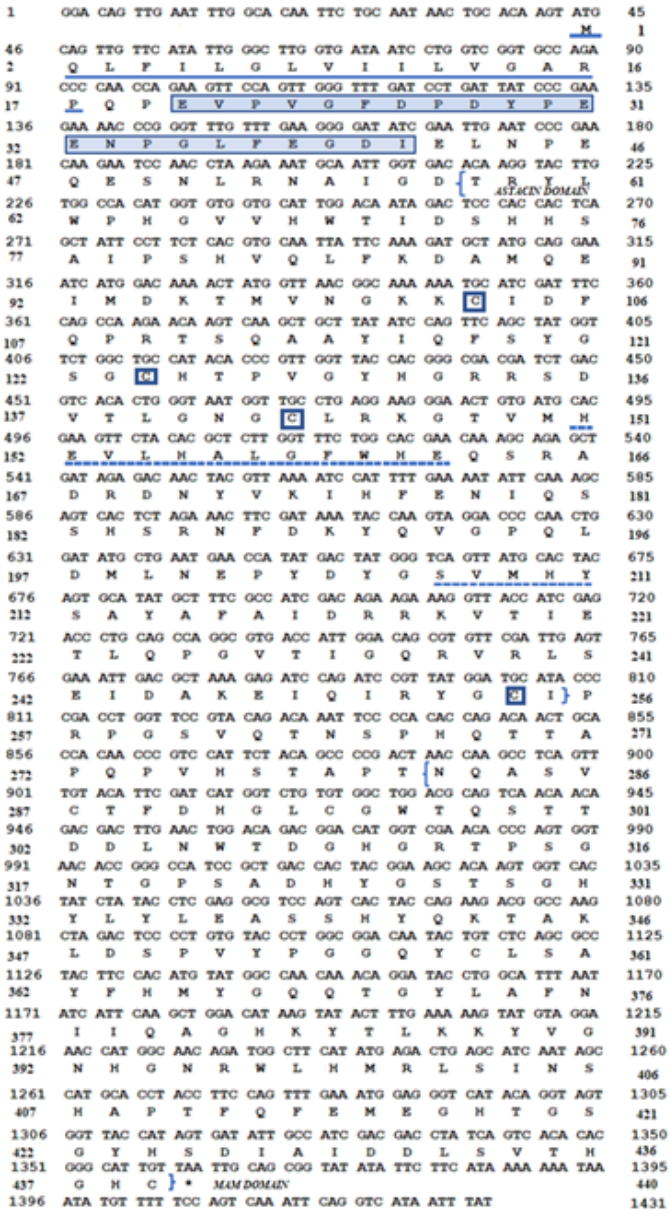

Fig 1: Complete cDNA sequence of *Magallana bilineata* Astacin like metalloproteinase (Mbi-ALMP) and its deduced amino acid sequence. Nucleotides are numbered from the first base at the 5' end. The signal peptide is underlined and the pro-peptide sequence is marked with the shaded box. All the conserved cysteine residues are boxed. The conserved zinc-binding motif and the met-turn are in dotted lines. The Astacin and MAM domain are marked in parentheses.

The catalytic domain possesses all the characteristic features of the Astacin family including the zinc binding catalytic site: "HEXXHXXGFXHEXX", the conserved met-turn "SVMHY" and four conserved cysteine residues at Cys103/Cys253 and Cys124/Cys143 which forms intradomain disulphide bridges. BLAST analysis revealed the unique structure of *Mbi-ALMP* comprising of a MAM domain as the only carboxy terminal domain. Many of the Astacin families have one or more non-catalytic domain at the carboxy terminal end associated with the protease domain. These include the EGF (Epidermal growth factor like), CUB (complementary subcomponents C1r/C1s), TRAF (Tumour necrosis factor receptor associated factor), MAM and MATH (meprin and TRAF homology) domains which are involved in functions such as interaction with regulatory proteins, anchorage to membrane, promote protein to protein interactions etc. It is the different non catalytic domains that yield different astacins with different enzymatic activities, regulation and expression [1, 23].

The MAM domain are found in several membrane proteins including meprins, hydra metalloproteinase 2 (HMP2), and enteropeptidases, where it is involved in molecular folding and oligomerization [26, 27, 8, 28]. MAM domains usually consists of 120-180 amino acid residues and has 14 conserved amino acids which includes 4 cysteine residues [1]. Astacin like metalloproteinases with a MAM domain as the only C terminal domain, similar to *Mbi-ALMP*, has been reported in squids *Heterololigo bleekeri* and *Todarades pacificus*. The physiological function of the MAM domain in these proteases (ALSM or Astacin like squid metalloproteases) is not yet established. Even though ALSM's are capable of hydrolysing myosin heavy chain, it is unlikely to be the true substrate of the enzyme due to its secretory nature [29]. Therefore, further studies are required to identify the true substrate of these unique Astacin proteases and elucidate its physiological function within the organism.

Sequence similarity comparison of the protease domain of *Mbi-ALMP* with that of other Astacin family members shows the highest similarity to the Astacin like protein of *C. gigas* and Nas-4 like protein of *Crassostrea virginica*, with an amino acid sequence identity of 100% and 91% respectively. This was followed by 68% sequence identity with Astacin like protein of *M. yessoensis*. However, all these three protein sequence records are a result of data generated using gene prediction methods from genomic sequence and have not yet been identified in vitro. Amino acid sequence identities with protease domains of Astacin like metalloproteinases from squids *S. maindroni, H. bleekeri* and *T. pacificus* ranged between 40-45%. A phylogenetic analysis (figure 2) constructed using MEGA software confirms the above findings and assigns *Mbi-ALMP* to the cluster of other Astacin like metalloproteinases from bivalves. Figure 3 shows the alignment of protease domain of *Mbi-ALMP* with selected known sequences of astacin members.

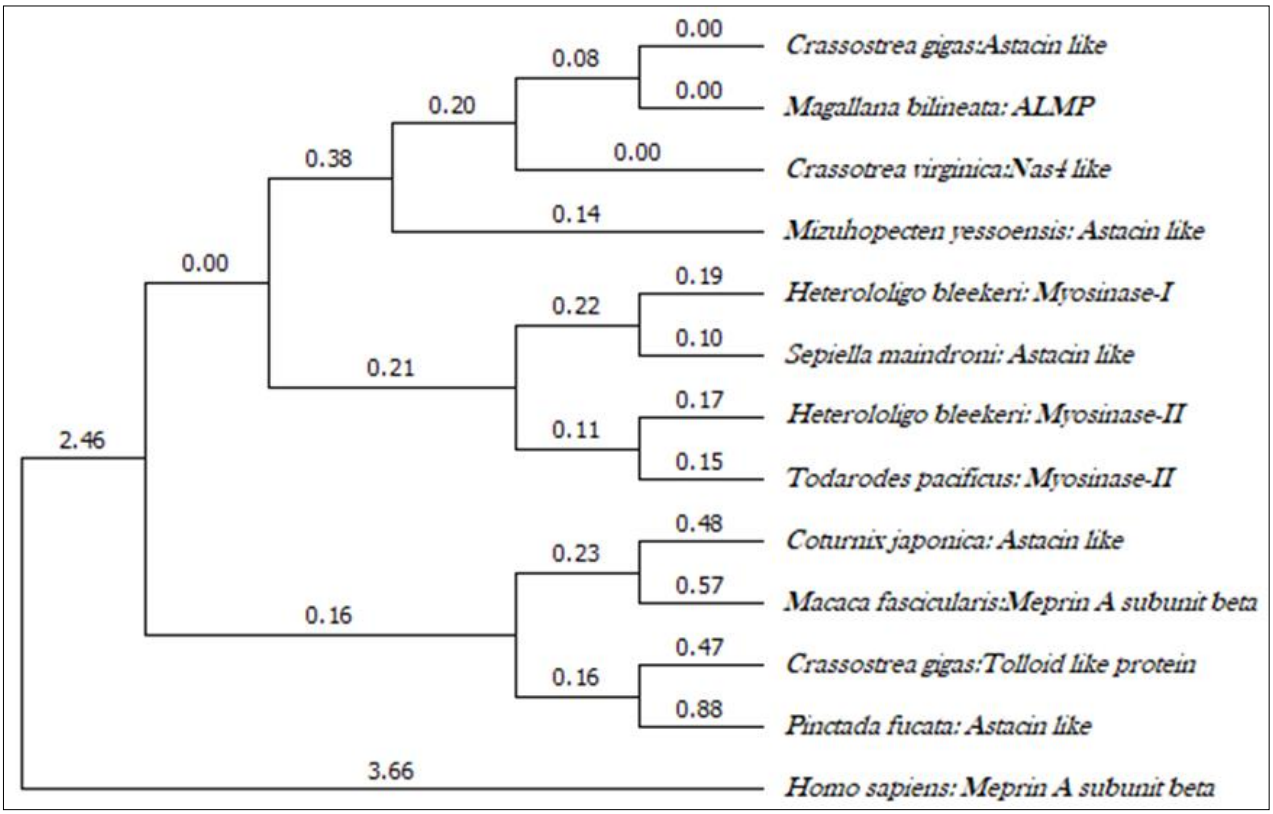

Fig 2: Phylogeny of the Astacin family based on sequence alignment of amino acid sequence of protease domains. The TREE was constructed using software MEGA v 7.0.21 using Neighbour joining method. The sequences used were *Crassostrea gigas*: astacin like (XP_011415612.1); Crassostrea virginica: Nas-4 like protein (XP_022318777.1); *Mizuhopecten yessoensis*: astacin like (XP_021341865.1); *Todarodes pacificus*: myosinase-II (BAC16239.1); *Heterololigo bleekeri*: myosinase III (BAC16240.1); Sepiella maindroni: astacin like (AGF33810.1); Heterololigo bleekeri: myosinase I (BAC16237.1); Crassostrea gigas: tolloid-like protein (CAD66417.1); Pinctada fucata: astacin metalloendopeptidase (AAX56337.1); Coturnix japonica: astacin like (P42662); *Macaca fascicularis*: meprin A subunit beta partial (EHH58837.1); Homo sapiens: meprin A subunit beta isoform1 precursor (NP005916.2).

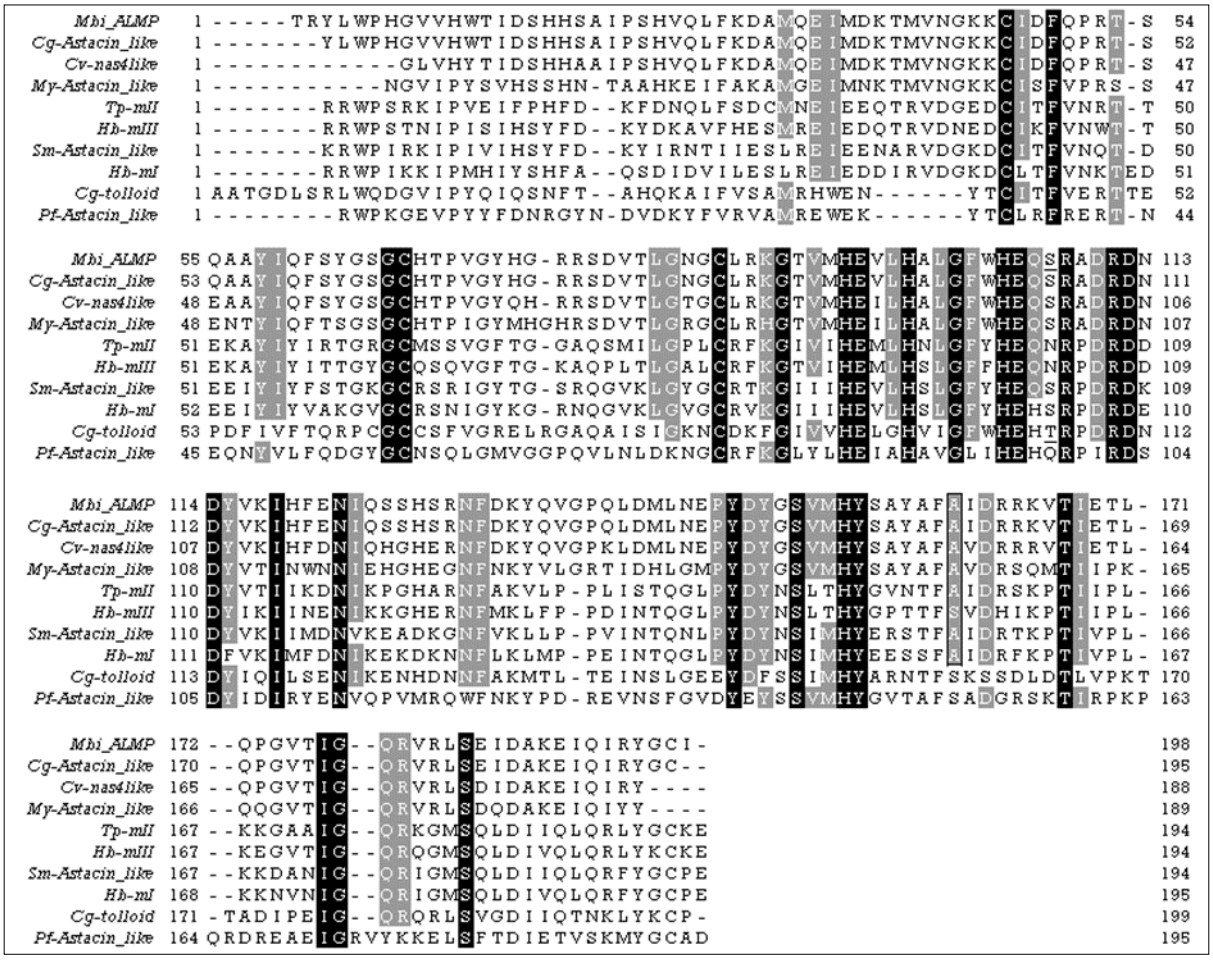

Fig 3: Comparison of amino acid sequences of protease domain of Mbi-ALMP with selected known sequences of *Astacin members*. Mbi-ALMP, *Magallana bilineata* Astacin like metalloproteinase; Cg-Astacin like, *Crassostrea gigas* Astacin like (XP_011415612.1); Cv-nas4like, Crassostrea virginica Nas-4 like protein (XP_022318777.1); My-Astacin like, *Mizuhopecten yessoensis* Astacin like (XP_021341865.1); Tp-mII, *Todarodes pacificus* myosinase-II(BAC16239.1); Hb-mIII, *Heterololigo bleekeri* myosinase III (BAC16240.1); Sm-Astacin like, Sepiella maindroni Astacin like (AGF33810.1); Hb-mI, Heterololigo bleekeri myosinase I (BAC16237.1); Cg-tolloid, Crassostrea gigas Tolloid-like protein (CAD66417.1); Pf-Astacin like, Pinctada fucata *Astacin metalloendopeptidase* (AAX56337.1).

4. Conclusion

In conclusion, we have cloned and identified the first Astacin like metalloendopeptidase *Mbi-ALMP* from the Indian oyster, *M. bilineata*. *Mbi-ALMP*, isolated from the digestive tissue of the oyster is a secretory protein with an Astacin catalytic domain and a MAM domain. Further studies are required to understand the structure, physiological function and regulation of these proteases.

5. Acknowledgments

The authors would like to thank the Director, CMFRI, Cochin, for providing the facilities and the DST-INSPIRE for financial support for undertaking this work.

6. References

1. Bond JS, Beynon RJ. The astacin family of metalloendopeptidases. Protein Science. 1995; 4(7):1247–1261.
2. Sonneborn HH, Zwilling R, Pfleiderer G. Evolution of endopeptidases. X. Cleavage specificity of low molecular weight protease from *Astacul leptodactylus* (Esch.). Hoppe-Seyler's Zeitschrift Fur Physiologische Chemie. 1969; 350(9):1097–1102.
3. Stöcker W, Gomis-Rüth FX, Bode W, Zwilling R. Implications of the three-dimensional structure of astacin for the structure and function of the astacin family of zinc-endopeptidases. European Journal of Biochemistry. 1993; 214(1):215–231.
4. Stöcker W, Zwilling R. Astacin. Methods in Enzymology. 1995; 248:305–325.
5. Geier G, Jacob E, Stöcker W, Zwilling R. Genomic organization of the zinc-endopeptidase astacin. Archives of Biochemistry and Biophysics.1997; 337(2):300–307.
6. Xiong X, Chen L, Li Y, Xie L, Zhang R. Pf-ALMP, a novel astacin-like metalloproteinase with cysteine arrays, is abundant in hemocytes of pearl oyster *Pinctada fucata*. Biochimica et Biophysica Acta - Gene Structure and Expression. 2006; 1759(11–12):526–534.
7. Geier G, Zwilling R. Cloning and characterization of a cDNA coding for Astacus embryonic astacin, a member of the astacin family of metalloproteases from the crayfish *Astacus astacus*. European Journal of Biochemistry. 1998; 253(3):796–803.
8. Sterchi EE, Stocker WW, Bond JS. Meprins, membrane-astacin metalloproteinases. Molecular Aspects of Medicine.2008;29(5):309–328.
9. Bond JS, Rojas K, Overhauser J, Zoghbi HY, Jiang W. The structural genes, MEP1A and MEP1B, for the α and β subunits of the metalloendopeptidase meprin map to human chromosomes 6p and 18q, respectively. Genomics. 1995;25(1):300–303.
10. Ge G, Greenspan DS. Developmental roles of the BMP1/TLD metalloproteinases. Birth Defects Research Part C: Embryo Today: Reviews. 2006; 78(1):47–68.

11. Katagiri C, Maeda R, Yamashika C, Mita K, Sargent TD, Yasumasu S. Molecular cloning of Xenopus hatching enzyme and its specific expression in hatching gland cells. International Journal of Developmental Biology. 1997; 41(1):19–25.
12. Shibata Y, Iwamatsu T, Oba Y, Kobayashi D, Tanaka M, Nagahama Y *et al*. Identification and cDNA cloning of alveolin, an extracellular metalloproteinase, which induces chorion hardening of medaka (*Oryzias latipes*) eggs upon fertilization. Journal of Biological Chemistry. 2000; 275(12):8349–8354.
13. Yasumasu S, Yamada K, Akasaka K, Mitsunaga K, Iuchi I, Shimada H *et al*. Isolation of cDNAs for LCE and HCE, two constituent proteases of the hatching enzyme of *Oryzias latipes*, and concurrent expression of their mRNAs during development. Developmental Biology. 1992; 153(2):250–258.
14. Elaroussi MA, DeLuca HF. A new member to the astasin family of metalloendopeptidases: A novel 1,25-dihydroxyvitamin D-3-stimulated mRNA from chorioallantoic membrane of quail. Biochimica et Biophysica Acta (BBA) - Gene Structure and Expression. 1994; 1217(1):vii–viii.
15. Rawlings ND, Morton FR, Kok CY, Kong J, Barrett AJ. MEROPS: The peptidase database. Nucleic Acids Research. 2008; 36(1):320–325.
16. Gomis-Rüth FX, Trillo-Muyo S, Stöcker W. Functional and structural insights into astacin metallopeptidases. Biological Chemistry. 2012; 393(10):1027–1041.
17. Yang S, Wu X. Tolliod-like gene in *Crassostrea ariakensis*: Molecular cloning, structural characterization and expression by RLO stimulation. Fish and Shellfish Immunology. 2009; 27(2):130–135.
18. Hedgecock D, Gaffney PM, Goulletquer P, Guo XM, Reece K, Warr GW. The case for sequencing the Pacific oyster genome. Journal of Shellfish Research. 2005; 24(2):429–441.
19. Zhang G, Fang X, Guo X, Li L, Luo R, Xu F *et al*. The oyster genome reveals stress adaptation and complexity of shell formation. Nature. 2012; 490(7418):49–54.
20. Thomson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research. 1994; 22:4673–4680.
21. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution. 2013; 30(12):2725–2729.
22. Yokozawa Y, Tamai H, Tatewaki S, Tajima T, Tsuchiya T, Kanzawa N. Cloning and biochemical characterization of astacin-like squid metalloprotease. Journal of Biochemistry. 2002; 132(5):751–758.
23. Semenova SA, Rudenskaya GN. The astacin family of metalloproteinases. Biochemistry (Moscow) Supplement Series B: Biomedical Chemistry. 2009; 3(1):17–32.
24. Roberts S, Goetz G, White S, Goetz F. Analysis of genes isolated from plated hemocytes of the Pacific oyster, *Crassostreas gigas*. Marine Biotechnology. 2009; 11(1):24–44.
25. Kaushal GP, Haun RS, Herzog C, Shah SV. Meprin A metalloproteinase and its role in acute kidney injury. American Journal of Physiology-Renal Physiology. 2013; 304(9):F1150–F1158.
26. Beckmann G, Bork P. An adhesive domain detected in functionally diverse receptors. Trends in Biochemical Sciences. 1993; 18(2):40–41.
27. Kitamoto Y, Yuan X, Wu Q, McCourt DW, Sadler JE. Enterokinase, the initiator of intestinal digestion, is a mosaic protease composed of a distinctive assortment of domains. Proceedings of the National Academy of Sciences of the United States of America. 1994; 91(16):7588–7592.
28. Yan L, Fei K, Zhang J, Dexter S, Sarras MP. Identification and characterization of hydra metalloproteinase 2 (HMP2): A meprin-like astacin metalloproteinase that functions in foot morphogenesis. Development. 2000; 127(1):129–141.
29. Yokozawa Y, Tamai H, Tatewaki S, Tajima T, Tsuchiya T, Kanzawa N. Cloning and biochemical characterization of astacin-like squid metalloprotease. Journal of Biochemistry. 2002; 132(5):751–758.