Characterization of a serine/threonine protein phosphatase regulatory subunit in the Indian pearl mussel

Rekha Das, Chandan Debnath, Gopal Krishna, Annam Pavan Kumar, Hamari Debbarma and Sankar Prasad Das

Abstract
Reversible phosphorylation is a key mechanism for regulating the biological activities of functional proteins. We have partially characterized a serine/threonine protein phosphatase 6 regulatory protein from the mantle tissues of Indian freshwater pearl mussels *Lamellidens marginalis* and *Parreysia corrugata*. The characterized sequences showed more than 75% sequence identity at both the DNA and protein levels of the species. Importantly, the characterized sequences did not show similarity to any other genes in the homology search. A predicted 23 amino acid polypeptide stretch from both the species was 100% identical in all mollusc sequences available till date (across isoforms) and more than 95% similar to a wide range of species. The predicted protein folding patterns showed alpha helices separated by intervening loop regions in both the *in silico* translated polypeptides, similar to reported regulatory subunits of serine/threonine protein phosphatases. Considering the extent of structural similarity of this polypeptide stretch among the molluscs and the fact that the sequences were isolated from the mantle tissues, it may be speculated that the genes partially characterized here could be primarily involved in shell formation and bio-mineralization process.

Keywords: *Lamellidens marginalis*, *Parreysia corrugata*, bio-mineralisation, mantle

Introduction
Bio-mineralization is a complex set of processes for the formation of inorganic solids within a living system. Formation of nares is one such fascinating bio-mineralization process encountered in many molluscs. Central to the process of bio-mineralization are proteins that guide and regulate the formation and growth of the inorganic crystals. In mollusc shell and pearl, such proteins are collectively called shell matrix proteins (SMPs) [1]. Biological activity of key proteins is determined to a large part by the reversible phosphorylation in eukaryotic cells [2]. Reversible phosphorylation is known to affect protein function in a variety of ways such as inducing conformation changes on substrate proteins and creating docking sites for phospho-dependent protein interaction domains [3]. Indeed, erroneous phosphorylation and de-phosphorylation of SMPs have been shown to result in abnormally growing prismatic layers and nacre tablets with reduced crystallinity and possibly changed crystal orientation [1]. Therefore exploration of specific proteins involved in the reversible phosphorylation mechanism of SMPs warranted.

It is estimated that more than 98% of reversible phosphorylation in proteins occur in serine/threonine residues [2, 4] catalysed by specific classes of kinases and phosphatases that cause phosphorylation and de-phosphorylation respectively of specific target proteins. The serine threonine Protein phosphatases (PP) are classified on the basis of their substrate specificity and inhibitor sensitivity into type-1 and type 2 phosphatases [5]. One of the type-2 category phosphatases, namely PP-6, plays an important role in cell signalling and cell cycle [4]. Mutations that affected the binding of the catalytic subunit of PP6 (PP6c) to the regulatory subunits resulted in increased mitotic defects in human cells [6]. PP6 holoenzyme is thought to play an important part in DNA double strand breaks repair [7] and is reportedly indispensable for embryogenesis [8].

The evolution of highly conserved protein phosphatase enzymes has recently captured scientific attention owing to its significance in cell viability and functioning. It is now known that the diversity of functional PPPs in eukaryotic proteome is derived from the many
combinations of catalytic and regulatory subunits [9]. In other words the whole array of specific protein phosphatase complexes encountered in the eukaryotic cell system owes their specificity in function as much to the regulatory subunits as to the catalytic units. PP6 holoenzyme is described as a heterotrimer comprised on the PP6 catalytic subunit, a SAPS domain containing regulatory subunit and an ankyrin repeat-domain containing regulatory subunit (ARS) [10]. Endogenous PP6 regulatory subunit 3 (PP6R3) co-precipitated 50% of PP6 in cell extracts. In fact, PP6R3 alone was sufficient to bind to the catalytic subunit, indicating it is the major binding factor for the PP6 holoenzyme [11].

In this study, an attempt was made on partial characterization of the serine/threonine protein phosphatase 6 regulatory subunit 3 from the mantle tissues of Indian freshwater pearl mussels, Lamellidens marginalis and Parreysia corrugata. Considering that the mRNA was derived from the major site of shell bio-mineralization in the species, it is possible that the characterized PP6R3 subunits isoform could have direct implications on the mussel shell matrix protein functions.

Materials and methods
RNA isolation and cDNA synthesis
Total RNA was isolated from mantle tissue samples of Lamellidens marginalis (Class Bivalvia; Family Unionidae) and Parreysia corrugata (Class Bivalvia; Family Unionidae) using RNasy® Mini Kit (Qiagen, USA) following manufacturer’s instructions. The isolated RNA was reverse transcribed using R2DFast 1st strand cDNA synthesis kit® (GCC Biotech, India). The single stranded cDNA was subjected to PCR (F: primer; R: Primer) using touchdown thermal cycling conditions of 1 cycle of 94 °C for 5 min, 35 cycle of 94 °C for 1 min/57-52 °C for 1 min/72 °C for 1 min and one cycle of 72 °C for 5 min.

Sequencing and bioinformatics analyses
The amplified products were checked on a 2% agarose gel to check size specificity of the bands. The amplicons of expected size were excised from the gel, purified, and subjected to sequencing on a Genetic Analyzer 3500xl (Applied Biosystems, USA).

The sequences were subjected to homology search on NCBI BLASTn to confirm specificity. The partial cDNA sequences were aligned with homologous mollusc sequences available in NCBI Genbank that showed more than 60% similarity. Phylogenetic trees were constructed using Phylgeny.fr platform [12, 13]. Within the online assemblage platform of tools for alignment, alignment refinement, phylogeny and tree rendering, PhyML 3.1/3.0 aLRT software was employed for phylogeny using maximum likelihood method in this study. The secondary structure prediction of predicted polypeptides was performed using Phyre 2 server [14].

Results and Discussion
Bio-mineralization is a complex set of molecular processes. Phosphorylation of shell matrix proteins is known to be a major factor that affects bio-mineralization process in mollusc shells [1]. In fact, reversible phosphorylation is one of the major post transcriptional modifications in proteins that govern their function and turn over [2]. In this study, partial characterization of serine/ threonine protein phosphatase 6 regulatory subunit 3 from two Indian freshwater pearl mussels is reported. The single primer pair used to amplify the sequence from both the species was designed following a methodology developed by our laboratory (Unpublished data). The partial cDNA sequences of the target gene and their respective predicted amino acid sequences characterized from Lamellidens marginalis and Parreysia corrugata (NCBI Acc. No: MN518757 & MN518758 respectively) were subjected to BLASTn homology searches. The characterized sequences showed more than 75% sequence identity at both DNA and protein levels across the species. Nucleotide similarity of more than 60% satisfies the criteria for phylogenetic tree construction [15]. Fig. 1 depicts the phylogenetic tree constructed using homologous sequences available in public domain from related species.

It may be observed that the clustering of the sequences followed the expected taxonomic hierarchy, and our reported sequences showed relatively higher similarity with other bivalve species. Importantly, the characterized sequence did not show similarity to any other genes in the homology search indicating that this is a highly conserved stretch. The in silico translated polypeptide stretch “QQA FS DQ YLQQMTS NFIDQF GFN EEFQ EEEK A DS P FGDRISSI” from L. marginalis was identified as SIT4 Yeast associated protein (SAP) family by Interpro software, confirming the specificity of our reported sequences. The human PP6 holoenzyme was identified to be an ortholog of the budding yeast SIT4 protein, involved in cell cycle regulation [16, 17]. The highly conserved SAPs domain held by the regulatory subunits is known to be the site of interaction between the SIT4 protein in the yeast, for the formation of the functional holoenzyme [11]. In fact, the predicted 23 amino acid polypeptide stretch from both the species “QQA FS DQ YLQQMTS NFIDQFGFN” was 100% identical in all mollusc sequences available till date (across isoforms) and > 95% similar to a wide range of species. The predicted protein folding patterns showed alpha helices separated by intervening loop regions in both the in silico translated polypeptides (Fig.2.a, b).
The protein phosphatase 6 catalytic subunit interacts with the regulatory subunit of the PP6 holoenzyme. Further studies to identify the role of these novel malleable protein sequences in the process of shell biomineralization of the freshwater pearl mussel are warranted.

Acknowledgements

The authors are grateful to Department of Biotechnology, Government of India for funding this study under the Twinning Project Scheme (Project ID: BT/PR16977/NER/95/377/2015; PIMS code OXX04066). The authors gratefully acknowledge the Director, ICAR Research Complex for NEH Region, Umiam for kind support and providing the facilities for the conduct of this study. We also mention our special gratitude to Dr. Sebastien Santini for his dedicated management of the Phylogeny.fr platform.

References


Our characterized sequences showed significant similarity to reported homologous sequences from multiple mollusc species both at DNA and polypeptide levels. The in silico translated polypeptide stretch from the characterized sequences showed homology to the SIT4 Yeast associated protein (SAP) family proteins, which is known to be the site of interaction between the regulatory unit and the catalytic unit of the PP6 holoenzyme. Further studies to identify the role of these novel malleable protein sequences in the process of shell biomineralization of the freshwater pearl mussel are warranted.

Conclusion

We report the results of our study where we have partially characterized a serine/threonine phosphatase 6 regulatory protein from the mantle tissues of Indian freshwater pearl mussels Lamellidens marginalis and Parreysia corrugata.


