

Insights from the Shell Proteome: Biomineralization to Adaptation

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Abstract

Bivalves have evolved a range of complex shell forming mechanisms that are reflected by their incredible diversity in shell mineralogy and microstructures. A suite of proteins exported to the shell matrix space plays a significant role in controlling these features, in addition to underpinning some of the physical properties of the shell itself. Although, there is a general consensus that a minimum basic protein tool kit is required for shell construction, to date, this remains undefined. In this study, the shell matrix proteins (SMPs) of four highly divergent bivalves (The Pacific oyster, *Crassostrea gigas*; the blue mussel, *Mytilus edulis*; the clam, *Mya truncata*, and the king scallop, *Pecten maximus*) were analyzed in an identical fashion using proteomics pipeline. This enabled us to identify the critical elements of a “basic tool kit” for calcification processes, which were conserved across the taxa irrespective of the shell morphology and arrangement of the crystal surfaces. In addition, protein domains controlling the crystal layers specific to aragonite and calcite were also identified. Intriguingly, a significant number of the identified SMPs contained domains related to immune functions. These were often are unique to each species implying their involvement not only in immunity, but also environmental adaptation. This suggests that the SMPs are selectively exported in a complex mix to endow the shell with both mechanical protection and biochemical defense.

Key words: biomineralization, shell matrix proteins, calcification, calcite, aragonite, evolution

Introduction

Bivalves are the second most bio-diverse extant molluscan class after the gastropods (Bieler et al. 2014) with more than 9000 living species and they are characterized by a tremendous array of shell architectures. These shells play a key role in protecting bivalves from predators, pathogens and to some extent from other environmental conditions, such as desiccation, wave action and iceberg damage (Checa 1993; Harper et al. 2012). The shells are formed by a biologically controlled process (biomineralization), which results in a composite material that is made of approximately 95% calcium carbonate (CaCO₃) and between 1% and 5% organic components (Lowenstam and Weiner 1989). In nature, CaCO₃ exists as different crystal polymorphs such as aragonite, calcite, and vaterite, which along with crystal size and organic matrix endow these biomineralized structures with their unique physio-chemical properties (Harper 2000). It is of great interest to material scientists that the aragonite and calcite shells

formed by bivalves are very durable compared with the geological inorganic forms. For example, aragonite formed biologically by bivalves is 3000 times tougher than pure aragonite (Currey 1977). The CaCO₃ crystals in shells are arranged in layers with a distinctive pattern to form complex microstructures. So far, more than 30 different microstructures of bivalve CaCO₃ have been documented (Carter 1980).

In this regard, paleontological research has greatly aided our understanding of shell microstructure evolution. Bivalve fossil records date back to the Cambrian, although the first radiation event occurred in the Ordovician. There is substantive evidence that the primitive mineralogy was aragonite (Vendrasco et al. 2011) with clear patterns in the developing mineralogy associated with the seawater chemistry in which these organisms evolved (Wood and Zhuravlev 2012). The calcitic mineralogy is likely to have derived after the taxa evolved along with the changes in the global environment from the aragonitic-facilitating seas of the Ediacaran/early Cambrian to the subsequent calcitic seas (542–488 My)

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(Wood and Zhuravlev 2012). Modern bivalves secrete a range of both aragonite and calcite and the development of different mineralogies and structures is clearly a polyphyletic trait (Harper 2016). Why this complex pattern of evolution has occurred is still under debate, but may not be related to the different solubilities of the calcium polymorphs (Harper 2016).

It is commonly accepted that shell formation is biologically controlled through diverse molecules such as proteins, glycoproteins, lipids, and carbohydrates (Gouletquer and Wolowicz 1989). The majority of the research to date has focused on the shell matrix proteins (SMPs) and sugar moieties present in the shell matrix space. SMPs play a significant role in CaCO₃ crystal nucleation, crystal growth and in generating the different crystal polymorphs even though they constitute only a very minor proportion of the shell matrix (Wheeler et al. 1981; Falini et al. 1996). SMPs are secreted in the mantle, an epithelial tissue lining the shell and are exported to the shell matrix space (Addadi et al. 2006).

Proteomic research investigating SMPs has been hampered by the lack of genomic resources in these non-model organisms. These data, namely, genome and transcriptome sequences, are needed to provide the cDNA transcripts or gene predictions, against which, the peptide fragments can be matched. Access to these sequences significantly enhances the chances of identifying meaningful functional information (Joubert et al. 2010). Initially, the mollusc SMPs were predicted from the genomic and transcriptomic data from mantle tissue (Miyamoto et al. 1996; Sudo et al. 1997) but this did not confirm their presence and incorporation into the shell matrix space. Next generation sequencing has enabled the rapid development of mantle transcriptome databases, with the resultant ESTs used as reference sequences to identify the peptides or proteins extracted from shell matrix space (Joubert et al. 2010; Berland et al. 2011). So far, SMPs from a variety of different mollusc species have been identified employing this approach, e.g., *L. gigantea* (Marie et al. 2013), *H. asinina* (Marie et al. 2010), *H. cumingii* (Berland et al. 2013), *P. margaretifera*, and *P. maxima* (Marie et al. 2012), *C. gigas* (Marie et al. 2011; Zhang et al. 2012), *M. edulis* (Marie et al. 2011; Liao et al. 2015) *M. galloprovincialis* (Gao et al. 2015) etc. Also, many groups have generated transcriptomic data from the mantle of different species, which could facilitate future shell proteomic studies (Jeong et al. 2007; Clark et al. 2010; Joubert et al. 2010; Shi et al. 2013; Artigaud et al. 2014; Freer et al. 2014). A preliminary outcome from analyses of these data is the recognition of the complexity of the molecular and structural diversity of the SMPs that are brought into play by the different species for growth and control of their intricately ordered shell structures, resulting in few shared proteins even within a taxon (Jackson et al. 2010).

The analysis of specific bio-mineralizing proteins in divergent taxa such as sea urchins, gastropods, and bivalves has revealed the evolutionary divergence and independent parallel evolution of these genes (Jackson et al. 2010). In spite of having similar morphological traits, the SMPs even from closely related bivalve clades show very different protein sequences with extensive domain shuffling. For example, the PIF

protein from *P. margaretifera*, *P. maxima*, *P. penguin*, and *M. galloprovincialis* show different primary amino acid sequences with diverse domain arrangements (Suzuki et al. 2009). However, some exceptions exist, such as the carbonic anhydrases, which are frequently found in the shell matrix and are highly conserved throughout the metazoans (Le Roy et al. 2014). Establishing a complete catalogue of proteins and their functions present in the shell matrix space is critical, not only understanding their role in an evolutionary context but also in terms of the constraints faced by the organism in their living environment and their provision of adaptive plasticity. With the latter becoming increasingly important with regard to understanding how shell microstructures impact on the plasticity of the shell and the ability of the organisms to cope with environmental change (Charrier et al. 2013).

In this study, the SMPs identified from three model species namely, the Pacific oyster (*Crassostrea gigas*), the blue mussel (*Mytilus edulis*), and the king scallop (*Pecten maximus*) are presented. These data are supplemented with the recently published proteomic data of the soft shell clam (*Mya truncata*) (Arivalagan et al. 2016) to enable a comparison of the SMPs of highly divergent species with the aim of uncovering an evolutionary conserved suite of shell proteins. These species are widely distributed in the North Sea and are of considerable economic importance in Europe. They differ in their evolutionary lineage, *modus vivendi* and shell structure. *M. truncata* (taxon: Heteroconchia) diverged from the other three species (taxon: Pteriomorphia) approximately 513 My ago (Plazzi and Passamonti 2010). Within the Pteriomorphia bivalves, *C. gigas* (clade: Ostreoida), and *P. maximus* (clade: Pectinoida) diverged from *M. edulis* (clade: Mytiloida) around 479 My ago. The divergence of the Ostreoida and Pectinoida is estimated to be 421 My ago (Ren et al. 2010) (fig. 1).

In terms of life style, *C. gigas* usually found in intertidal and sub-tidal zones, they settle on one another in large numbers and can even form reefs (Wrange et al. 2010). *M. edulis* lives in intertidal zones by attaching to any roughened, scarred or pitted surface by means of byssal threads (Seed 1969). *P. maximus* are free swimmers and live in relatively shallow waters. Unlike the other three models, *M. truncata* is burrower and lives 1–2 feet below the seabed and uses its siphon to filter microalgae. Also, the mineralogy and microstructure of these species differ significantly from one another. The *C. gigas* shell is purely calcite: the outer shell layers are calcite prisms, the middle shell layers are chalky calcite and inner shell layers are foliated calcite (Marie et al. 2011). In contrast, *M. truncata* shell is composed only of aragonite, with a granular microstructure in the outer layer, and cross-lamellar aragonite in the inner layer. Both *P. maximus* and *M. edulis* shells are a combination of both calcitic and aragonitic layers, but with differing organization. The *M. edulis* shell is composed of calcite prisms in the outer layer, with middle and inner layers of aragonite nacre. *P. maximus* has outer and inner layers of foliated calcite layers and a middle layer of cross-lamellar aragonite.

To date, the majority of the research on SMPs has been focused on their roles in the context of the biological control of shell formation, calcium biochemistry pathways, and

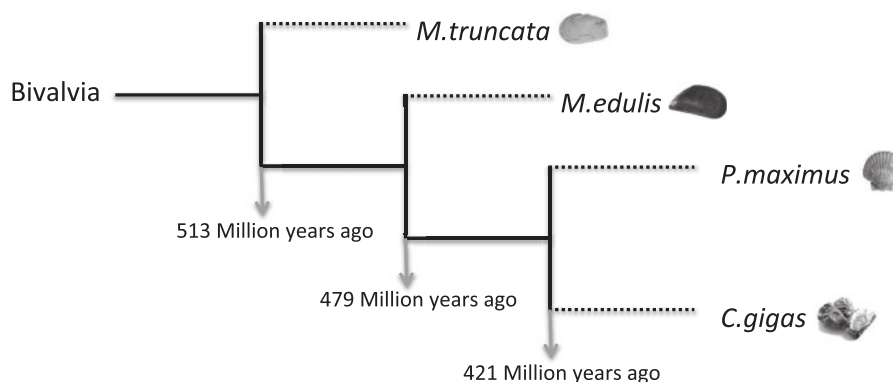


Fig. 1. Phylogeny of four bivalve models and their corresponding divergence times based on a Bayesian analysis using mitochondrial genes (Plazzi and Passamonti 2010; Ren et al. 2010).

crystal growth. However, the role of other proteins present in the shell matrix space has been ignored or at best considered as cellular contaminants. In this study, the shell matrix proteins of these four evolutionary divergent bivalves (*C. gigas*, *M. edulis*, *M. truncata*, and *P. maximus*) were extracted and analyzed in an identical fashion via proteomics pipeline. This enabled robust interrogation of the data to test the hypothesis that there is an ancient conserved “tool box” of calcification proteins and that proteins of varying functional domains are exported selectively to the shell matrix space to enhance successful species-specific adaptation.

Materials and Methods

Sample Preparation

Bivalve samples were collected at different locations in west coast of Scotland (supplementary fig. S1, Supplementary Material online). Proteomics experiment was carried out using the shells from the animals used for generating transcriptome data. Each shell was washed overnight with 5–10% sodium hypochlorite (NaOCl), to remove any organic impurities and the periostracum. Any remaining periostracum was removed by manual abrasion. The shells were then washed with water for an hour and air-dried. The shells were then cut and polished with a dremel tool. The cut shell pieces were washed briefly with NaOCl and then rinsed with water and air-dried. A mortar and pestle was used to grind the shell pieces into a powder, which was then graded using a 250 μm mesh.

Shell powders (3 g) were decalcified using 5% cold acetic acid for an hour and then left in 10% acetic acid overnight for the complete dissolution of CaCO_3 . The resulting liquid was centrifuged (14,000 rpm, 20 min, 4 °C) to separate the supernatant [acid soluble (ASM)] and the pellet [acid insoluble fractions (AIM)]. The AIM was washed five times with water, freeze-dried. The supernatant was concentrated and washed with water by centrifugation through a 10 kDa filter (Sartorius, VIVASPIN 20) to enrich the proteins from small molecules, such as peptides and other impurities. The filter containing the ASM proteins was then washed with water and freeze-dried. Prior to the digestion of the proteins, denaturation was performed, by treating both ASM and AIM samples with 30 μL of 8 M urea solution for an hour at 37 °C. Next

15 mM dithiothreitol (DTT) dissolved in 100 mM triethyl ammonium bicarbonate (TEAB) was added and an incubation at 37 °C performed for 1 h. Alkylation was performed by the further addition of 20 μL of 15 mM iodoacetamide, with the samples kept in the dark at room temperature for 1 h. After this alkylation step, trypsin was added to the AIM and ASM samples (8.5 and 5 μg , respectively) and incubated at 37 °C overnight. The samples were acidified with 10 μL of 10% formic acid and the digested peptides were desalted, by applying 40 μL of the digested matrix samples to 4 mm Empore™ SPE (Sigma-Aldrich, France) cartridges. The resulting peptides were quantified using the Bicinchoninic acid kit (Sigma-Aldrich, France).

Mass Spectrometry Analysis

One μg of the peptide sample was obtained by mixing both AIM and ASM samples and the mix was analyzed in a nano LC system (Dionex Ultimate 3000, France) coupled to a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, France). The injected digest was enriched on a 5 mm C_{18} column (5 μm , 100 Å pore, 300 μm i.d., LC packing France) and the peptides were separated on a 50 cm nano-column packed with C18 phase (3 μm , 75 μm i.d.) at a flow rate of 300 $\text{nL}\cdot\text{min}^{-1}$ using the following gradient: 1% solvent B (98% ACN, 0.1% formic acid) to 40% B in 180 min, 40% B to 60% B in 2 min, 60% B for 28 min. The mass spectrometer was operated in positive ion mode and the MS and MS/MS spectra acquired in the Orbitrap and linear ion trap, respectively. The parameters used for survey scans were 300–2000 m/z , resolution 30,000 AGC (automatic gain control) target 2×10^5 and maximum injection time 100 ms. CID fragmentation was performed for 20 of the most abundant precursors with the following parameters: minimum intensity 500, isolation window 2 Da, normalized collision energy 35%, AGC target 5000, and maximum injection time 100 ms, dynamic exclusion was enabled (repeat count 1, duration 80 s).

Data Analysis

MS/MS spectra were searched against the six-frame translated mantle transcriptomic databases of *P. maximus*, *M. edulis*, and *C. gigas* from British Antarctic Survey (Yarra

et al. 2016) using in-house Mascot server (Matrix Science, London, UK; version 2.4.1) These data were supplemented by additional protein sequences of *C. gigas* from Uniprot (<http://uniprot.org>, last accessed April 2015). The following parameters were used for the database search: carbamidomethylation of cysteine as a fixed modification and oxidation of methionine and de-amidation of aspartic acid and asparagine as variable modifications. The mass tolerance for MS and MS/MS experiments was set as 10 ppm and 0.5 Da respectively. Protein identification results from Mascot were further validated using Scaffold software (version 3.6.5, Proteome Software Inc., Portland, USA). Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm. Protein identifications were validated if they could be established at greater than 95.0% probability (assigned by Protein Prophet algorithm) and matched at least two unique peptides, each containing minimum eight amino acids. False discovery rate was estimated to be <1% by the scaffold software.

BLAST2GO tool (version 3.3) was used to carry out sequence similarity protein searches against the NCBI database. Interspecies comparison of the SMPs from each species was carried out using locally installed NCBI BLAST tool version 2.4.0 (Blast-p) (<ftp://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/>, last accessed March 2016) with SEG filtering disabled. Circos (<http://circos.ca/>- version 0.69, last accessed March 2016) was used to represent the BLAST-p results. Conserved domains were detected using Simple Modular Architecture Research (SMART) domain prediction tool (<http://smart.embl.de/>, last accessed March 2016).

Results and Discussion

Although the literature is abundant with lists of genes and proteins identified from the mantle tissue and also the shell matrix space of different species, a robust comparison, in terms of the number and nature of the identified proteins, is difficult due to the different extraction and analytical techniques employed. Therefore, in this study, protein extraction, peptide generation, and data analysis were carried out under same conditions, thus enabling a much more reliable comparative study of SMPs from the four different species and investigation of a potentially ancient shell-building molecular tool kit. The shell proteome of *M. truncata* was recently published by our group (Arivalagan et al. 2016) wherein we employed the same analytical workflow as described in this work, which enables us to compare the shell proteome of the four species. It is still under debate as to whether the shell building mechanism is conserved in molluscs. Previous molecular phylogeny and paleontological studies have identified multiple origins of nacre evolution within the Mollusca (Jackson et al. 2010; Vendrasco et al. 2011) and this is merely one of the possible components of a mollusc shell. To date, the existence of an ancient conserved shell building molecular mechanism within the bivalves remains unexplored. In this study, we specifically chose to carry out an inter-species comparison

of molluscs that exhibit a range of shell mineralogy and microstructures to gain insight into the conservation of SMPs.

55, 46, and 46 different SMPs were identified in the shell matrices of *C. gigas*, *M. edulis*, and *P. maximus*, respectively (supplementary tables S1 and S2, Supplementary Material online) and in case of *M. truncata* 67 SMPs had been identified in a recent study (Arivalagan et al. 2016) (supplementary table S3, Supplementary Material online). It is worth to note that proteins reported are inferred from the peptides detected in MS/MS experiments and potentially represent a part of the shell forming proteome. The SMPs were searched against NCBI-nr database using BLAST-p program in order to find similar proteins with known functions. 24 similar proteins ($\approx 48\%$ of the identified SMPs) were retrieved in *P. maximus*, 16 in *M. edulis* ($\approx 34\%$), 31 in *M. truncata* ($\approx 46\%$) and 48 in *C. gigas* ($\approx 87\%$). The higher number of similar proteins was found in *C. gigas* is due to the availability of an annotated genome. The number and extent of sequence similarities between the identified SMPs varies with specific-species comparisons (fig. 2). A high degree of matches was found between *C. gigas* and *P. maximus* and the least number of significant matches was obtained in comparisons of *M. edulis* to the other species. In general, the results are consistent with evolutionary divergence times except for *M. edulis*. This was surprising, as based solely on the principle of gene conservation over evolutionary time, it was expected that *M. edulis* would share greater number of similar proteins with *C. gigas* and *P. maximus* since they are closer in divergence times. However, *M. truncata* actually shared more proteins with *C. gigas* and *P. maximus* than *M. edulis*. This result cannot be explained purely by the differences in the crystal structure of the shells of the different species (Hedegaard and Wenk 1998; Chateigner et al. 2000), as the *C. gigas* shell is completely calcite, whereas that of *M. truncata* is completely aragonite. *M. edulis*, which is a mix of aragonite and calcite, would be expected to share significant protein homologies with both.

Several other factors might be contributing to this surprising finding, which relate to environmental factors and the innate highly plastic adaptation strategies of *Mytilus* species. For example, *M. edulis* species exhibit a high degree of tolerance to freezing and to variations in salinity (Williams 1970). Several populations of *M. edulis* have shown rapid adaptation to changes in their environment (temperature, salinity, ocean acidification, availability of food, etc.) over only a few generations (Widdows 1978; Vuorinen et al. 2002) by altering not only their shell structure but also their life history traits, such as, shell size, life span and reproductive behavior. In addition, *M. edulis* has several hybridization zones with *M. trossulus* and *M. galloprovincialis* in the Atlantic Ocean, the Baltic, and North seas (Gardner 1996; Riginos and Cunningham 2005). These factors might have exerted selective pressure on the shell proteins in *M. edulis* to evolve several times in parallel.

In spite of the considerable differences in SMPs between the four species, a set of shared SMPs was identified between the four species, which indicate the existence of a shell-forming “molecular tool box” inherited from their common ancestors and potentially conserved among the class Bivalvia for the purpose of building a calcified shell (Marin et al. 2007).

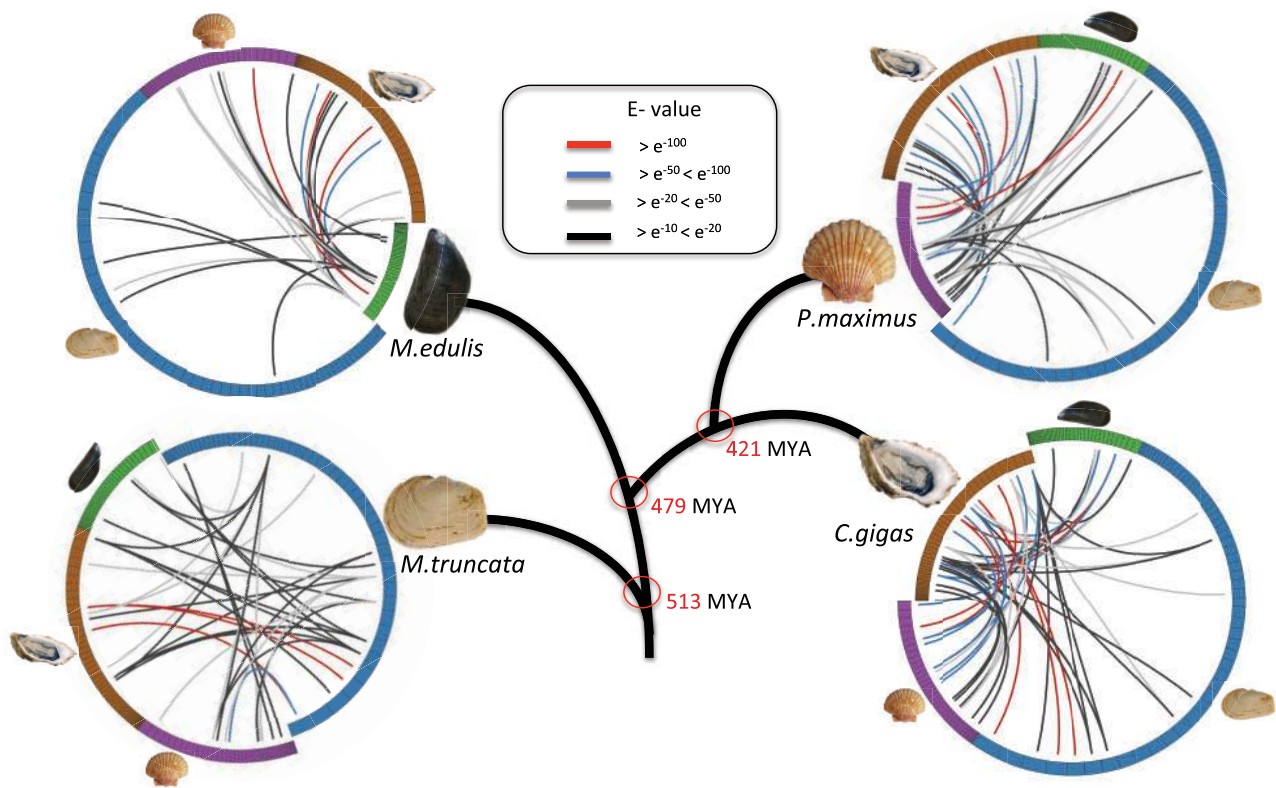


Fig. 2. Circos representation of the sequence similarities of identified SMPs from the four bivalve models (cut-off E -value $\geq e^{-10}$). Colored lines represent similarity scores, with the corresponding E -values denoted in the figure.

Although, BLAST sequence similarity analysis enabled the identification of longer cDNA transcripts within the transcriptome, matching the peptide fragments isolated in this study, the functional annotation of such transcripts was incomplete. In order to detect multiple domains within the same protein from divergent evolutionary sources, a domain prediction tool was used to provide additional annotation and further compare the SMPs in the context of their functions (Schultz et al. 1998, 2000). The final annotations were binned into different functional groups and are discussed in detail below.

Biocalcification—the Basic Tool Kit

This study identified proteins with functional domains that are common to the shell matrix space of all four species (fig. 3). Thus, irrespective of shell morphology and microstructure, we suggest that these four domains are evolutionarily maintained and represent part of the “basic tool kit” for the construction of the CaCO_3 molluscan exo-skeleton. These domains are tyrosinase, carbonic anhydrase, chitin binding-2, and Von Willebrand factor-A.

The main function of the tyrosinase domain in invertebrates is sclerotization, particularly, the arthropods. In this process, insoluble protein polymers (Nagai et al. 2007) are formed by transforming tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), which is the same mechanism employed in the formation of periostracum in bivalves (Zhang et al. 2006). Tyrosinase has undergone multiple lineage-restricted expansions, even in the closely related bivalve super families

Ostroidea (*Crassostrea*) and Pterioidea (*Pinctada*) clades (Aguilera et al. 2014). Similarly, in the shells of the four species under investigation, tyrosinases of different lengths and domain arrangements were identified (supplementary fig. S2, Supplementary Material online). *C. gigas* and *P. maximus* had paralogous tyrosinase domain-containing proteins and it has been hypothesized that these two species may have evolved these paralogs to perform two distinctive functions during shell formation (Nagai et al. 2007). In the case of *P. maximus* and *M. edulis*, one protein with two-tyrosinase domain repeats was identified, but the functional significance of such tyrosinase repeats is yet to be discovered.

Carbonic anhydrase (CA) belongs to the metallo-enzyme superfamily; it plays a key role in catalyzing the reverse hydration of carbon dioxide during calcium carbonate formation. Earlier experiments on these domain-containing proteins documented their role either in calcification (Marie et al. 2008) or as an inhibitor of the calcification process (Nacrein) (Miyamoto et al. 1996). In the present study, this domain differed in sequence length between species. However, in spite of this difference the conformation and activity of carbonic anhydrase in the biocalcification process is suggested to be highly conserved in metazoans (Le Roy et al. 2014).

Chitin binding 2 (ChBt-2) and Von Willebrand factor type-A (VWA) domains are extra cellular matrix domains usually found together in SMPs, such as PIF, the acidic matrix protein first identified in *Pinctada fucata*, Blue mussel shell protein (BMSp), etc. The main role of the ChBt-2 domain in the SMP is to interact with chitin as this is the key component of the

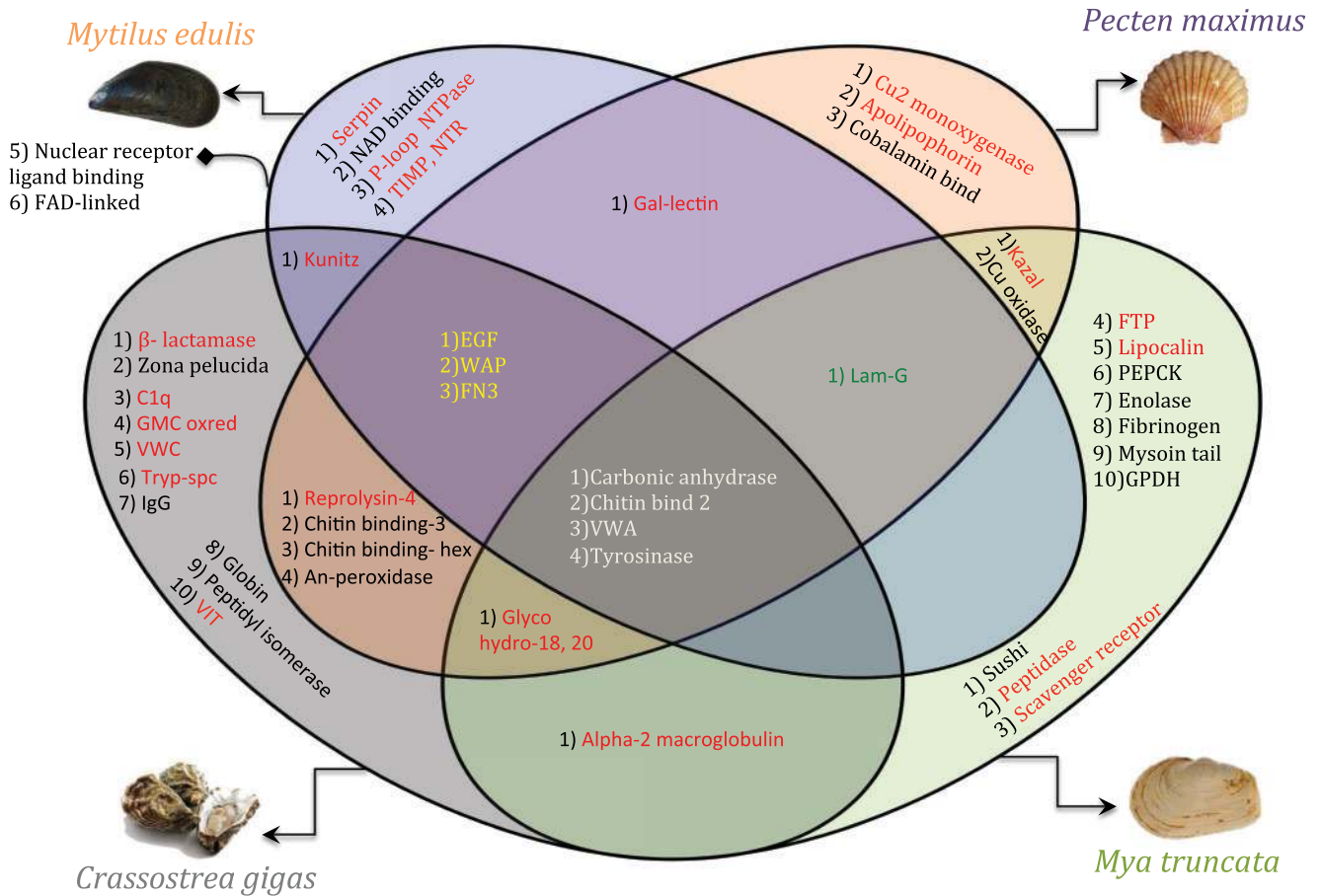


Fig. 3. Venn diagram of the protein-domains identified from the four bivalve SMPs. Domains common to the four bivalve models are indicated in white. Domains in yellow and green are related to calcite and aragonite shell layers. Immunity-related domains are represented in red.

mollusc organic matrix (Mann 1988). The VWA domain is usually found in glycoproteins and exhibits an adhesion function through protein–protein interactions (Whittaker and Hynes 2002). These domains are also components of hydrophilic glyco-proteins, which usually assemble to form silk-like protein complexes (Weiner 1979), which play a key role in chitin-scaffolding and in arranging CaCO_3 crystals to such scaffolds.

Crystal Lattice Associated Proteins

As indicated earlier, all the four species have different layers of CaCO_3 polymorphs in their shells. Apart from the basic tool kit, a subset of protein domains was identified that was common to the shells with similar crystalline layers and these domains potentially influence the specific nucleation or arrangement of particular polymorphs of CaCO_3 . Three domains were observed in shells containing a calcite layer: epidermal growth factor (EGF), fibronectin-3 domains (FN3), and whey acidic protein (WAP) domains. These were absent in *M. truncata*, which has a shell made entirely of aragonite entirely (fig. 3).

The EGF domain is a calcium-binding motif and comprises 45 amino acids arranged in two anti-parallel β -sheets with several cysteine residues (Stenflo et al. 2000). The EGF-like domain has been reported previously in shell matrix proteins, but the authors noted that this protein only occurred in the

prismatic (calcitic) layers but not in the nacreous layer of *Pinctada* shells (Marie et al. 2011). EGF domains are always found in the SMPs as tandem repeats and this repeating design is favored in any system during evolution due to its stability against enzyme attack, with the repeating structures forming compact dimeric or tetrameric pseudo-symmetrical structures (Miller et al. 2001) which is thought to help in the compact arrangement of calcite crystals. Although the Lam-G domain is specific to shells with aragonite layers, intriguingly it exhibits a function similar to EGF (Panayotou et al. 1989). This may be due to the presence of 8 cysteine repeats that show homology to EGF, which contains six cysteine repeats (Beck et al. 1990), implying that these domains have an important role in specific crystal nucleation events and arrangements.

The extracellular matrix adhesive glycoprotein, fibronectin (Donaldson and Mahan 1983) is also only identified in shells made of calcite layers. Hanein et al (1993) demonstrated the affinity of fibronectin for calcite in the presence of water molecules (Hanein et al. 1993). Thus fibronectin might help in specific arrangement of calcite crystals in the hydrogel scaffold.

WAPs are highly conserved proteinase inhibitor domains found in a whole range of species, from arthropods to mammals. WAP domains in lustrin A and perlwapin in the nacre of gastropods *H. laevigata* and *H. asinina* play major roles in shell formation (Shen et al. 1997; Treccani et al. 2006; Marie et al.

2010). Treccani et al. (2006) proposed that WAP domains inhibit crystal growth in the fast growing *c*-axis and thus form a platy geometrical aragonite crystal. They also reported strong interactions between perlwapin and calcite crystals (Treccani et al. 2006). The WAP domains identified in previous proteomic studies were always discussed in relation to nacre or aragonite formation. However, WAP domains have also recently been observed in fully calcite eggshells (Mann K and Mann M 2015; Rose-Martel et al. 2015). Our observations support this latter finding and it is highly likely that WAP domains are also involved in calcite formation.

Immunomodulatory Proteins

In the context of shell formation, the identification and presence of a large number of proteins containing immunity-associated domains is puzzling. On the one hand their presence in the shell matrix space seems to reinforce the notion of the protective function of the shell through the use of additional biochemical pathways. How this actually contributes to the overall defense of the organism needs further investigation. Out of 47 separate functional domains identified in this study, 21 domains ($\approx 45\%$) could be related to immunity function (fig. 3). This observation may raise questions with respect to this group of proteins potentially being contaminants from the mantle tissue. Proteins such as, actins, tubulins, and myosins, which are the most common and abundant proteins in cellular tissues, can be found in proteomics studies of the shell matrix space, but they are considered to be cellular contaminants (Marie et al. 2013; Ramos-Silva et al. 2013). However, none of these highly abundant structural proteins were identified in the shell extractions described here. This verifies the efficiency of the shell cleaning and protein extraction procedures. In addition, some of the domains with immunity functions were species-specific, which suggests environmental adaptation. These immunity-domain proteins are described in more detail below.

Proteases and Protease Inhibitors

Proteases are enzymes that perform proteolysis and are found to be highly expressed in haemocytes during microbial invasions (Gerdol et al. 2011). The peptidase domain was found to be unique to *M. truncata* SMPs, whilst the trypsin-like serine protease (Tryp-Spc) domain was unique to *C. gigas*. The metalloprotease domain repolysin is a highly conserved disintegrin domain and is associated with the inflammatory response. This domain is present in both *C. gigas* and *P. maximus*. Although the disintegrin domain has been described earlier in the sea urchin genome (Angerer et al. 2006), this is the first report of its occurrence in bivalves. Out of the four gigasin family proteins identified in *C. gigas*, only gigasin-6 contains β -lactamase domain and could be classified as a protease in accordance with the earlier reports (Marie et al. 2011). This suggests that gigasin-6 might be an inactivated form of lactamase-related proteins and may exhibit protease functions.

Protease inhibitors are enzymes, which inhibit proteases released by pathogens. Several protease inhibitors such as metallo-proteinase inhibitors including the tissue inhibitor

of metalloproteinase (TIMP), inter-alpha-trypsin inhibitor (VIT), and serine protease inhibitors containing serpin, kunitz and kunitz domains were identified in this study. TIMP and serpin were unique to *M. edulis* and have been previously reported in the haemocytes and tissue of the gastropod *Haliotis discus discus* (Bathige et al. 2015), *Crassostrea gigas* (Montagnani et al. 2001), and the clam, *Tegillarca granosa* (Wang et al. 2012). Although, TIMP has been identified previously in *C. gigas* hemocytes and associated tissues, this protein was not identified in this study. This result may be due to the fact that even though many immunity proteins are secreted, only a few are selectively exported to the shell matrix space. The VIT domain was found uniquely in *C. gigas*, and has previously been identified in the haemocytes of *B. glabrata* gastropod mollusc (Mittra et al. 2005) with an immunity function. Serine protease inhibitors with domains such as kunitz were present in both *P. maximus*, *M. truncata*, with kunitz domain proteins present in *M. edulis* and *C. gigas*. So far, SMPs containing both kunitz and kunitz domains have not been reported in the same species. Therefore, the presence of either one of these serine protease inhibitors in the shell matrix space might indicate an adaptation trait.

Alpha-2-macroglobulin (A2M) was identified in both *C. gigas* and *M. truncata*, and belongs to a class of protease inhibitor that inhibits wide variety of endopeptidases, which was first discovered in humans (Family I39, MEROPS peptidase database) (Rawlings et al. 2016). However, the mode of action of A2M in invertebrates is similar to that of found in humans and has been shown to interact with a wide variety of proteases and thus may be used as a defense against several infections caused by bacteria, parasites and pathogens (Armstrong et al. 1996). A2M is the part of the ancient innate immune system and this idea is supported by homology to complement factors C1, C3 and C5 (Armstrong et al. 1998).

Pattern Recognizing Receptors and Associated Proteins

Pattern recognizing receptors (PRR) are proteins involved in the initial sensing of infections and are found in plants and animals. They are present in epithelial, endothelial, and fibroblasts, which recognize the pathogens through pathogen associated molecular patterns (PAMPs) (Takeuchi and Akira 2010). Lipid binding proteins, lectins, toll-like receptors (TLR), nod-like receptors (NLR) etc. all fall under this category and are associated with the innate immune response (Gordon 2002).

With the exception of *M. edulis*, we detected at least one lipid-binding domain in the other species. *C. gigas* had a complement component (C1q) domain-containing protein, which belonged to the C1q protein family. A bacterial challenge experiment using *Listonella anguillarum* in the scallop *Chlamys farreri* showed significant increases in the levels of C1q proteins in haemocytes, kidney and gills, confirming their participation in immunity related functions (Zhang et al. 2008; Gerdol et al. 2011). Lipocalin and the scavenger receptor (SR) are lipopolysaccharide-binding domains, which were uniquely found in *M. truncata*. The SR domain recognizes gram positive bacteria and is involved in the immune

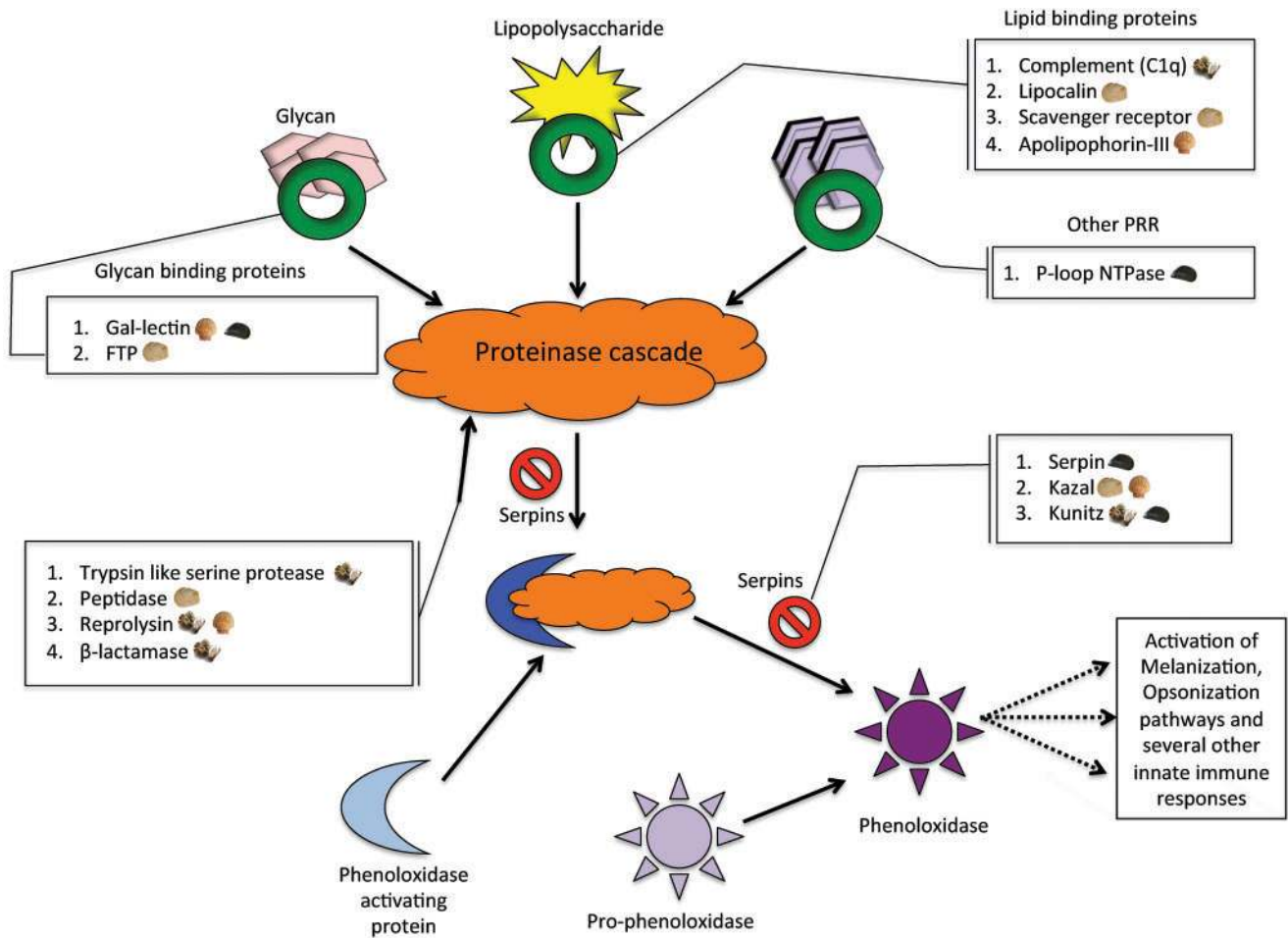


Fig. 4. Representation of the phenoloxidase oxidation pathway in arthropods adapted from Jiravanichpaisal et al. (2006). The boxed annotations show the identified functional domains and their corresponding protein families in this activation pathway.

signaling cascade (Fabriek et al. 2009). Lipocalins are multi-functional domains and have been shown to be recruited for various biological functions such as coloration, complement fixation, etc. as well as regulation of the inflammatory response (Greene et al. 2001). *P. maximus* SMPs had matches to an apolipoprotein-III, a lipid-binding domain, and an immune stimulating protein factor. Previous experiments have shown the former domain to participate in the detoxification of lipopolysaccharide endotoxins, involvement in interactions with gram positive bacteria and also lysozyme activity (Weers and Ryan 2006).

The Gal-lectin proteins identified in *P. maximus* and *M. edulis*, and the FTP domains found in *M. truncata* SMPs are lectins that recognize carbohydrate moieties. Lectins are glycoproteins used for pattern recognition and defense responses (Bachère et al. 1995). The increased expression of lectins in hemocytes or tissues during disease or in stressed conditions has previously been reported in bivalves (Kang et al. 2006).

In *M. edulis*, a P-loop containing NTPase domain was identified, which has not been reported to date in any bivalve shells. This domain along with leucine rich repeats are usually found in the metazoan nucleotide oligomerization domain (NOD)-like receptors (Koonin and Aravind 2002). The P-loop NTPase mediates self-oligomerization in the presence of ATP.

This domain shows a high specificity for pathogens and parasites and thus significant involvement in the innate immunity in molluscs (Yeretssian et al. 2008).

Glycohydro-18 and 20 domains are functional domains of chitinase or chitinase and were found in proteins of *C. gigas*, *P. maximus*, and *M. truncata*. The main role of these domains is to degrade chitin polymer to dimers and subsequently to monomers (Joshi et al. 1989). Chitinase and chitinase-like proteins are triggered by PRRs when they recognize the chitin cell wall of pathogens, particularly fungi (Vega and Kalkum 2012). Interestingly, chitinase is also involved in the remodeling of shells (Yonezawa et al. 2016).

Immunity Cascades and SMPs

Phenoloxidase is an important precursor in the several biochemical cascades of innate immunity pathways in invertebrates such as melanization, phagocytosis, capsulation, opsonization etc. (Söderhäll and Cerenius 1998; Cannon et al. 2004). In the phenoloxidase pathway (fig. 4), organic molecules such as glycans, lipopolysaccharides are assembled by lectins, lipid binding proteins and PRRs along with proteinases to form a proteinase cascade. These bind to the phenoloxidase activating protein to form a complex that converts prophenoloxidase to phenoloxidase. Serpins act as an

inhibitor and regulate this cascade. Intriguingly, all the identified SMPs containing immunity domains in the four species are part of the phenoloxidase pathway indicating selective export of these SMPs to the shell and the critical importance of this immune response pathway to bivalve molluscs.

Proteins with No Predicted Functions

In addition to biomineralization and immunity-related proteins, there were also proteins identified which had domains with no predicted functions. For example, *C. gigas* had two unique proteins, one with six repeats of a Von Willebrand factor-C (VWC) domain and another with a glucose-methanol-choline (GMC) oxidoreductase domain. Previously, single VWC domain containing proteins have been discussed in the context of immunity in crustaceans and arthropods (Sheldon et al. 2007; Chen et al. 2011), so the presence of a protein with six repeats of the VWC domain may indicate an immune function. Similarly the GMC domain is a highly conserved domain and has been shown to play a significant role in the development and immunity of insects (Iida et al. 2007). However, the role of these domains in the immune response of bivalves has not been demonstrated to date and they could also represent novel proteins, with, as yet, unknown functions.

Many of the proteins identified contain one or more low complexity domains (LCDs) in addition to the functional domains noted above. These LCDs confer the proteins with enhanced flexibility. This can be advantageous as during the transport of proteins across the cell membrane, proteins with flexible conformations pass more readily than the folded proteins (Wright and Dyson 1999). As the SMPs are secreted and exported from the mantle, low complexity regions may aid in their transportation via epithelial cell membranes of the mantle. The intrinsic plasticity conferred by the LCDs could also allow a single protein to recognize several biological targets without sacrificing its specificity (Wright and Dyson 1999). Low complexity regions tend to evolve rapidly via mitotic slippage or meiotic recombination events (Marcotte et al. 1999) and could be an environmental adaptation response (Verstrepen et al. 2005).

Conclusions

Our data describes for the first time, the components of an evolutionary conserved bivalve “shell forming proteome tool kit” that are involved in shell calcification processes. This investigation takes advantage of the development of a standardized extraction and analytical proteomic pipeline, which was applied to four divergent bivalve species (*C. gigas*, *M. edulis*, *P. maximus*, and *M. truncata*), exhibiting different shell mineralogies and microstructures. This work enabled the identification of SMPs that are specific to aragonite and calcite mineralogies. Proteins specific to a single species were hypothesized to be adaptive to their *modus vivendi*, especially as these were most often identified in the blue mussel, *M. edulis* that inhabits highly variable environments and is subject to frequent inter-species hybridization events. Of significant note was the fact that numerous immunity-related domains were identified,

which may aid in defense against pathogens. Thus shells are endowed with both embedded biochemical defense mechanisms and mechanical protection. This newly uncovered complexity of SMPs may underpin the evolutionary processes, which led to the successful diversification of the bivalves. Work is now in progress on the different elements of this basic tool kit to determine how these conserved proteins interact to produce the myriad of shell complexity observed in extant bivalves.

Supplementary Material

Supplementary figures S1 and S2 and tables S1–S3 are available at *Molecular Biology and Evolution* online.

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