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The biocalcification of mollusk shells and coral skeletons: Integrating molecular, proteomics and bioinformatics methods

Sequeira dos Ramos Silva, P.

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Chapter 6

Conclusions and Perspectives

The research described in this thesis is focused on biominerals of calcium carbonate, in particular the organic matrix embedded in the mineral phase of mollusk shells and corals skeletons, which directly controls the biocalcification process in these organisms. In particular, the organic matrix proteins are considered as key components of the biological control over mineralization. Since the first report by Miyamoto and co-workers of a protein (Nacrein) from the mollusk shell of the pearl oyster in 1996 [53], many proteins in shells and other structures were identified by a one-per-one approach using classical molecular biology and biochemistry techniques. Until recently, the existing information was manageable and consisting of some proteins with very specific signatures: secretion signals, acidic domains, carbonic anhydrases, low complexity regions, and not much else.

With the entrance in the post-'omic' era, the fields of genomics, proteomics and transcriptomics also reached the biomineralization field and have allowed the scientific community to discover a much wider range of proteins taking part in the control of biocalcification mechanisms. These large newly identified protein datasets, are often difficult to interpret in light of the current models of calcification. The main goal of this dissertation is to give a contribution in this context. Apart from identifying several new proteins by means of high-throughput technologies, we present a careful analysis of the results and further interpretation from functional and evolutionary perspectives.

In Chapter 2 we made use of a previous proteomic experiment on the nacre of the freshwater mussel *Unio pictorum* to discover a novel shell protein. Upsalin is a 12 kDa protein that has a complete new primary structure without similarities with any previous protein found in shells or other mineralized structures. Moreover, the purified Upsalin did not interfere with the morphology of calcite crystals *in vitro*, in spite of being present in the nacre and prismatic layers. These findings call the attention to two main aspects of organic matrix proteins. Firstly, the existence of many new proteins, "orphan proteins", retrieved from high-throughput technologies and needing to be characterized by new methods. Secondly, the fact that not all the proteins of the organic matrix are there to interact directly with the mineral phase but rather to interact with the other macromolecules in the extrapallial space.

In Chapter 3 we gave a significant contribution to the study of coral calcification by identifying thirty-six skeletal organic matrix proteins from the staghorn coral *Acropora millepora* - a species from the Great Barrier Reef. Unlike mollusks that have seen their lists of calcifying proteins growing significantly in the post-'omic' era, the discovery of organic matrix proteins in corals is still in its infancy. This work is one of the first combining proteomics with nucleic acid datasets to identify the "biomineralization toolkit" in corals. It is the first to give attention to a careful cleaning of the skeleton samples, retrieving exclusively extracellular regions of proteins. Among the 36 skeletal proteins we identified not only acidic molecules but also proteins from the extracellular matrix, enzymes, galaxins, orphan proteins and one toxin. This confirms what has already been shown for mollusks, in that the organic matrix proteome consists of an amalgamate of proteins with diverse functions. Substantial evidence is given to support the hypothesis that the proteins

may act in the extracellular calcifying medium either by being secreted or by remaining attached to the cell membrane. In the latter case, their extracellular region would actively participate in the biomineralization process, being subsequently cleaved by proteases and occluded within the newly formed skeleton. This proposed mechanism is new to coral calcification and it challenges the concept of an organic matrix formed only by secreted proteins and the hypothesis that biomineralization is occurs far from the cells.

Finally, we compared the *Acropora* skeletal proteome with three cnidarian genomes (*Nematostella vectensis*, *Hydra magnipapillata*, *Acropora digitifera*) and other skeletal proteomes by making use of several bioinformatic tools for sequence comparison. The results suggest a complex scenario for the evolution of skeletogenesis in scleractinian corals: we give evidences that the skeletal organic matrix proteins evolved multiple times within Cnidaria, by mechanisms of co-option and more broadly within metazoan, by domain shuffling. Evolutionary aspects of coral calcification have been poorly addressed mainly due to the lack of molecular markers. In this chapter we contributed with a set of proteins that can be used for further functional, evolutionary and environmental studies on coral calcification.

In Chapter 4 we dealt with the reliability of proteomic approaches applied to coral skeletons and other mineralized structures in metazoans. This is a very delicate issue that needed to be addressed sooner or later. Several high-throughput approaches applied to mineralized tissues in the recent years have retrieved a large number of unexpected proteins that, in light of the current knowledge on biomineralization mechanisms, should be considered as contaminants but are not treated as such. Thus our contribution in this context is to alert scientists studying

biomineralization, in particular the proteins, about the need of a careful cleaning of biomineral samples.

In Chapter 5 we went back to the model *Acropora millepora* to characterize the morphology and microstructure of its skeleton and to provide an overall biochemical and functional characterization of the organic matrix. This study complements the proteomic approach described in Chapter 3 and strengthens the specie *Acropora millepora* as a new emergent model to study biomineralization. Moreover we provide more evidences that part of the organic matrix proteins remain attached to the calicoblastic cell membranes, we show that the organic matrix of this coral has several specific features relative to other biomineral matrices and we observe that it has a strong interaction with calcite crystals *in vitro*.

To summarize, this thesis describes the characterization of 37 proteins involved in $CaCO_3$-biomineralization and sheds light on the mechanisms by which they govern biomineral formation. In addition, we provide new molecular markers for calcification studies. We show that there is a great variety of proteins composing the organic matrix having a strong acidic character, enzymatic domains, and adhesion or even putative antibacterial properties. On the other hand there are also novel proteins, for which the primary sequence or even further experimental characterization, like in the case of Upsalin, are not sufficient to affiliate them to a specific role in biomineralization. In order to elucidate their functions, a promising approach is to develop different gene silencing strategies applied to calcifying organisms. This can be indeed a very good tool to understand the functions of biomineralization proteins at organismal level. Such methods have been used by

Suzuki and collaborators to elucidate the role of Pif in the formation of ordered nacre tablets [125] whereas in a more recent article Wilt *et al*. show that inhibiting the gene of the spicule matrix protein SM30 - an abundant protein found occluded and surrounding sea urchin spicules - has no effect on larval spicule formation [260]. Therefore, simply preventing the expression of a certain gene may also not give definite answers on the function of novel biomineralization proteins. Promising technologies such as genome editing in which targeted gene(s) can be inserted, replaced or removed from the chromosomes are being applied in models such as human cells [261,262] or zebrafish [263,264], and can provide in the near future a powerful tool to explore the role of many proteins in other animals [265,266].

Beyond the unknown putative role of orphan proteins, other questions raise up from this dissertation. A central question is "how important and specific are each one of the organic matrix proteins for biocalcification". Indeed some of the proteins occluded in biominerals may be important but having redundant functions, *i.e.* easily replaceable by other organic matrix proteins. On the other hand we have identified proteins that are ubiquitously present in other tissues of the organisms and are therefore not biomineral-specific. Even though, an essential function in the biomineralization process should not be dismissed.

Another question emerging from Chapter 4 is "in which extent are the proteins of the organic matrix bound to the mineral phase". In fact, it is possible that more proteins could be removed with even harsher cleaning procedures than the ones carried out throughout the thesis. This hypothesis should be verified in the future by comparing multiple cleaning methods on the same biomineral (either by

extending the exposure of the cleaning agent or reducing the grain size of the mineral powder), followed by a proteomic analysis.

Finally, a central question that prevails is "how these proteins interact with each other in the calcifying organic matrix and with the biomineral itself". Indeed, the ongoing discovery of new proteins provides the basis to speculate about their interactions based on their sequences. However, this is not sufficient to uncover all possible scenarios of protein-protein and protein-mineral interactions. One would have to use either experimental or numerical methods to determine their 3D structures, and thus take advantage of these data to develop molecular dynamics or particle based simulations to fully predict those organic-mineral interactions.

Our aim in the near future is to be able to answer these and many other questions.