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Comparative analysis of the soluble organic matrix of axial skeleton and sclerites of *Corallium rubrum*: Insights for biomineralization

J. Debreuil, S. Tambutté *, D. Zoccola, N. Segonds, N. Techer, D. Allemand, É. Tambutté

Centre Scientifique de Monaco, Avenue Saint-Martin, MC-98000, Monaco

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ABSTRACT

We analysed the soluble organic matrix (SOM) of two biominerals formed by the same organism but differing by their morphological characteristics: the axial skeleton and the sclerites of *Corallium rubrum*. The results of 1D SDS-PAGE electrophoresis show for the two biominerals that SOM proteins bands have similar apparent molecular weight but differ in quantity. Further analysis by 2D electrophoresis reveals each protein band as a line of spots with different isoelectric points. Our results suggest that each SOM protein band consists of a mix of proteins and/or one unique protein with post-translational modifications. By immunohistochemistry, we show that antibodies raised against the SOM of axial skeleton and sclerites label the SOM of the two biominerals but also label the insoluble organic matrix suggesting the presence of common epitopes between the two biominerals and the two organic fractions.

1. Introduction

Biomineralization is the dynamical and physiological process by which organisms transform ions in a solution into a solid structure: the biomineral. Biominerals are found in all kingdoms of life. In addition to being mostly composed of inorganic compounds, some biominerals also contain organic molecules which are collectively termed “organic matrix” (for review on the general definition of the biomineralization process see Lowenstam, 1981; Lowenstam and Weiner, 1989; Mann, 2001). The organic matrix which is obtained after demineralization, is usually described as being composed of two fractions: the soluble organic fraction (soluble organic matrix = SOM) and the insoluble organic fraction (insoluble organic matrix = IOM) where solubility/insolubility refer to the medium in which demineralization occurs. In the literature, when considering these two fractions, it is suggested that the IOM plays a role as structural proteins forming a framework for crystal growth whereas the SOM plays a role in nucleation and crystal growth (Allemand et al., 1998; Belcher et al., 1996; Cui et al., 2007; Falini et al., 1996; Marin and Luquet, 2004) or in kinetics for ion supply (Tambutté et al., 2007b). It should be noted that due to the difficulty in re-solubilising IOM, most studies on organic matrix only deal with the biochemical characterization of the soluble part of organic matrix.

Characterization of the soluble organic matrix is well advanced for some biomineralizing model taxa, such as molluscs or echinoderms, where data are available from the ultrastructural to the biochemical and molecular levels (see Marin et al., 2008; Mann, 2001). For other organisms, data are far less abundant in spite of their importance for a better basic understanding of the biomineralization process. This is the case for Cnidaria among which scleractinian reef-building hexacorals are considered as the most important mineralizing group of the phylum and typically known for their role in producing tropical coral reefs. This is also the case for various other taxa such as octocorallian species from the genus *Corallium* (Gorgonacea, Coralliidae). Indeed both Scleractinia and *Corallium sp.* produce a calcium carbonate skeleton but species belonging to the genus *Corallium* also produce another calcium carbonate biomineral, the sclerites. Sclerites are distributed within the tissues and their morphology and morphometry have been described in detail by means of light and scanning electron microscopy (Weinberg, 1976). Among the different *Corallium sp.*, one is particularly culturally and economically important, the precious coral *Corallium rubrum* (Linnaeus; Fig. 1A) from the Mediterranean Sea. Indeed *C. rubrum* has been harvested for jewellery purposes for centuries (Allemand, 1993; Ascione, 1993; Liverino, 1983; Morel et al., 2000). However, some populations of *C. rubrum* are affected both by climate change (Garrabou and Harmelin, 2002; Harley et al., 2006; Harmelin, 2004) and local over-harvesting (Garrabou and Harmelin, 2002; Garrabou et al., 2001; Tsounis et al., 2006, 2007; Rossi et al., 2008) and quantification of the decline in populations of *C. rubrum* is still a matter of debate (Bussoletti et al.,

* Corresponding author.
E-mail address: stambutte@centrescientifique.mc (S. Tambutté).

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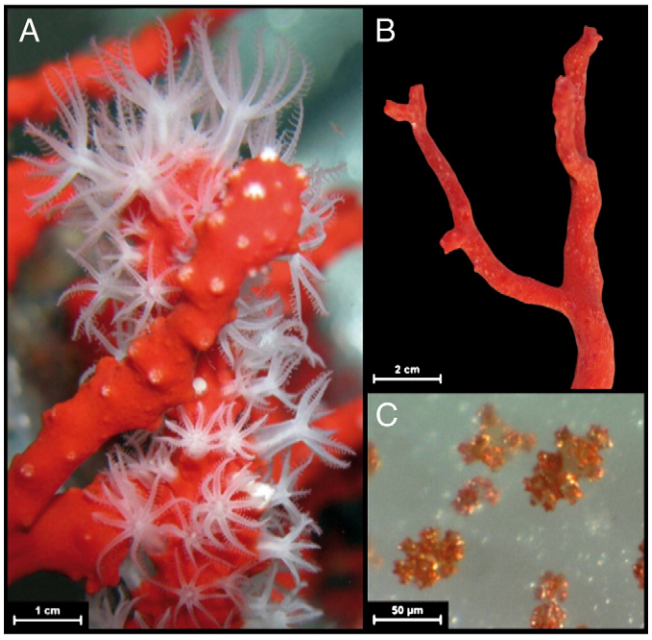

Fig. 1. (A): Living colony of *Corallium rubrum* with polyps (in white). (B): Axial skeleton of *C. rubrum*. (C): Sclerites of *C. rubrum*.

2010; Bruckner, 2009, 2010; Santangelo and Bramanti, 2010; Tsounis et al., 2010).

First studies on the biomineralization of *C. rubrum* were performed by Lacaze-Duthiers who concluded that the axial skeleton (Fig. 1B) results from the migration and fusion of sclerites (Fig. 1C) (Lacaze-Duthiers, 1864). This hypothesis was based on the observation that the axial skeleton was covered of micro-protuberances which had a similar shape as the sclerites. More than one century later, by combining histological observations (Grillo et al., 1993) with growth dynamics experiments (Allemand and Grillo, 1992; Allemand and Bénazet-Tambutté, 1996), it has been shown that the axial skeleton results first from the fusion of sclerites at the apex of the colony and then from an extracellular concentric secretion by the skeletogenic epithelium (see also review of Allemand, 1993). The sclerites are initially formed in intracellular vesicles within scleroblasts present in the mesoglea (Grillo et al., 1993). However it is not clear once they are released in the extracellular medium whether or not they continue growing as suggested for the gorgonian *Pseudoplexaura flagellosa* (Goldberg and Benayahu, 1987).

Axial skeleton and sclerites are very different in shape and size. The axial skeleton has a shrubby shape and can reach sometimes more than 50 cm in height and several centimeters in diameter (Liverino, 1983) whereas the sclerites have a microstructure which never reaches more than a few micrometers in axial or lateral directions (~30–50 µm). Sclerites are present in high amount within the mesoglea, about 10^6/mg tissue proteins (Allemand and Grillo, 1992). These two skeletal structures are composite material with a similar inorganic fraction of calcium carbonate crystallized under the form Mg-calcite and an organic fraction called "organic matrix" (OM). Based on multiscale physico-chemical characterization of the axial skeleton, it has been proposed that an organic matrix is present on length scales from nano-to macro scales and could control crystallisation, growth and hierarchical organization of the axial skeleton (Vielzeuf et al., 2008). From biomimetic experiments, it is suggested that the organic matrix plays a role of assemblers between inorganic building blocks forming mesocrystals (Cölfen and Antonietti, 2005) which have been evidenced in red coral (Vielzeuf et al., 2008, 2010).

Despite the high commercial value of its coloured skeleton and recent studies on its crystallographic architecture (Vielzeuf et al., 2008, 2010), very few data are available concerning the biochemical composition of its skeletal parts and especially the composition of its organic matrix which contains proteins, glycosaminoglycans and proteoglycans (Allemand et al., 1994; Borelli et al., 2003; Dauphin, 2006) as well as pigments such as carotenoïds (Merlin and Delé, 1983; Cvejic et al., 2007) or trans-polyacetylene molecules (Fritsch and Karampelas, 2008). Dauphin (2006) performed a comparative study of the organic matrix of the axial skeleton from *C. rubrum* and *Corallium johnsonii* but did not investigate sclerites. Concerning the organic matrix of other octocorals, the most abundant literature concerns alcyonarians such as *Lobophytum crassum* and *Synularia polydactyla* (Rahman and Isa, 2005; Rahman et al., 2005, 2006a,b, 2008; Rahman and Oomori, 2008) or *Leptogorgia virgulata* (Kingsley et al., 1990, 1996; Kingsley and Watabe, 1983, 1989; Samata et al., 1989) but these studies were only performed on sclerites since most of Alcyonacea do not possess a hard axial skeleton. When considering the organic matrix of axial skeleton, biochemical data are only available for scleractinian corals (for review see Tambutté et al., 2007a). To date, aside from the work of Allemand et al. (1994), no biochemical comparative analysis of OM has ever been made between two biomineralizing structures formed by a single Cnidarian species.

Comparative studies between different animal models or species are essential to achieve a better understanding of the biomineralization process. Furthermore, studying the process of biomineralization within the same organism which produces two different calcitic biominerals is also of interest since it provides a unique opportunity to determine different and common points in the machinery of the biomineralization process.

Our aim was to perform with the same analytical tools, a comparative biochemical study of the sclerites and axial skeleton of *C. rubrum*. After demineralization, we worked on the proteins of the soluble organic matrix (SOM) of each skeletal structure. In a first step, we characterized and compared the proteins of the SOM by 1D and 2D gel electrophoresis. Then we determined the site of organic matrix synthesis and the pattern of the organic matrix in the two biominerals by immunohistochemistry.

2. Materials and methods

2.1. Biological material and separation of skeletal structures

Colonies of *C. rubrum* (Linnaeus) (Anthozoa, Octocorallia, Gorgonacea, and Coralliidae) between 5 and 7 cm length were collected at 30 m depth in Marseille, Riou Island (Mediterranean coast of France). Samples were rinsed several times with filtered natural seawater (0.22 µm) and ultrapure water to remove mucus and potential contaminants. Then samples were incubated in 20% sodium hypochlorite (NaOCl) in order to eliminate soft tissues and separate sclerites from axial skeletons. Axial skeletons were removed from the bleach solution which was centrifuged in order to separate the soft tissues (in solution) from the sclerites. Axial skeletons were rinsed with ultrapure water and incubated in 1% sodium dodecyl sulphate (SDS) to remove potential residual sclerites. Then each skeletal structure was thoroughly rinsed with ultrapure water, freeze dried and observed under a binocular microscope to check for cleanness of the preparation. Sclerites and axial skeletons were separately cryo-ground (Spex SamplePrep 6770 apparatus) into powder of homogeneous granulometry (about 30 µm diameter). The powders were incubated with 2% NaOCl at 4 °C for 24 h to remove potential contaminants such as endoliths. The resulting solutions were centrifuged (3500 g, 5 min, 4 °C). The pellets of powders were rinsed several times with ultrapure water and freeze dried.