



# The Lysosome Origin of Biosilica Machinery in the Demospongiae Model *Petrosia ficiformis* (Poiret, 1789)

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The silicification mechanism in sponges is a biologically controlled process where the complex and amazing shape of spicules is the result of the hierarchical assembly of silicon particles to form a composite structure with organic compounds, mainly constituted by proteins. In this work, using an integrated approach of transcriptomic and proteomic analysis, we describe the protein content of sponge spicules in the marine demosponge *Petrosia ficiformis* (Poiret, 1789). Proteins from spicules were obtained via an ammonium fluoride extraction procedure to remove the inorganic silica followed by SDS-PAGE electrophoresis. The resulting data of LC-MS/MS analysis of the extracted SDS-PAGE bands were then processed with the MASCOT software to search against a database consisting of transcripts and predicted proteins of *P. ficiformis*. The results revealed a very heterogeneous group of 21 proteins, including silicatein beta, different isoforms of cathepsins, proteins with strong homologies with enzymes like sulphatases, glycosidases, lipid-related proteins, phosphatases, and some others with unknown function. Most of the proteins found here have structures and domains attributable to lysosomes enzymes and for this reason it could be related to these cellular structures the evolutionary origin of the biosilica machinery in sponges.

**Keywords:** marine sponges, *Petrosia ficiformis*, biosilica, spicules, lysosomes, silicatein, cathepsins

## INTRODUCTION

Sponges are very simple and ancient animals, but they have a remarkably complex genetic and molecular organisation (Sebé-Pedrós et al., 2018; Musser et al., 2021). The presence of sponge fossils from more than 600 million years makes palaeontologists consider this phylum as the oldest animal group still living on the earth (Erwin et al., 2011). Sponges are pluricellular organisms with a simple body structure not organised in specialised tissues or organs (Simpson, 1984). Nevertheless, the evolution from unicellular to multi-cellular animals needed a relevant implementation of gene regulation mechanisms (Brunet and King, 2017). Amazingly, there are many molecular analogies between the highly evolved multi-cellular animals and the simple multi-cellular sponges. The presence of RNA based regulatory factors like long noncoding RNA (Gaiti et al., 2018), or signal

cascade translation systems based on inflammatory pathway response (Pozzolini et al., 2016; Pozzolini et al., 2017) and growth factors (Pozzolini et al., 2019) are examples showing the great molecular similarities between sponges and other higher animals. The simplicity of sponges' body, the direct contact of their cells with the external environment (sponges filter water that permeates in all the body, through a very well organised channels system) and their sessile status induced on the other hand the need to protect from micro-organisms infections and from other animals' predation. This requirement is managed by the production of a great amount and variety of natural compounds, mainly supplied by symbiotic microorganisms (Brinkmann et al., 2017). All these features make sponges "unique" in the animal kingdom: a fascinating balancing of body simplicity and molecular complexity.

One of the most peculiar aspects of sponges is the structural organisation of their skeleton. The sponge body is supported by organic extracellular matrix (mainly collagen or/and "spongins" in some cases mixed with chitin) (Pozzolini et al., 2012; Ehrlich et al., 2018; Jesionowski et al., 2018) and in many species by inorganic structures called spicules, formed by amorphous silicon dioxide or, in Calcispongiae, calcium carbonate (Simpson, 1984; Ehrlich et al., 2011). Siliceous spicules have different shapes and dimensions with an extremely ordered organisation at a nanometre level (Şen et al., 2016). Biosilica is produced by Hexactinellida, by many Demospongiae and by some Homoscleromorpha (Simpson, 1984). There are many studies on the structural organisation and chemical composition of the biomaterial forming sponge spicules (see for overview Schoeppler et al., 2017; Wysokowski et al., 2018; Görlich et al., 2020; Pisera et al., 2021) and at present time, it seems clear that the features and the properties of biosilica depend on the composition of this material. Sponge biosilica is, in fact, a composite material where organic biomolecules contribute to its synthesis in environmental conditions, to the shape determination of spicules and to their technological properties (Khrunyk et al., 2020), but it is not easy at all to study the dynamics of spicule building. To satisfy this need, a multidisciplinary approach is necessary. Advanced material science technology to analyse in detail the structure and the properties of biosilica and good animal and cellular models to explain the molecular and genetic rules of spicule building are both necessary. At present, Hexactinellida have been mainly studied by material scientists: the production of large and long spicules (up to 2 meters long in *Monoraphis chuni*) (Müller et al., 2007; Ehrlich et al., 2008; Pisera et al., 2021) and their intriguing technological properties as optic fibre (Cattaneo-Vietti et al., 1996), were the main reasons for the choice of this class of animals. Demospongiae are instead approached by sponge cell biologists and molecular biologists, for the possibility to manage in controlled environment living animals and their sponge cell culture (Valisano et al., 2006; Pozzolini et al., 2014). It is also now clear that Hexactinellida and Demospongiae composite silica-based spicules apparently have common features but also very relevant differences in their composition (Müller et al., 2011; Shimizu et al., 2015). Definitely, many questions are still open

to understand exhaustively the biological rules of biosilica production. Specifically, in Demospongiae, siliceous spicules are most commonly formed by monoaxonic or tetraxonic needle-like structures, from some  $\mu\text{m}$  to 1-2 mm long (Hooper and Van Soest, 2002). In the Demospongiae *Tethya aurantium* was for the first time sequenced and cloned silicatein, one key protein involved in the biosilicification process (Shimizu et al., 1998; Cha et al., 1999). In this last two decades, many studies approached from different points of view the role of silicatein in Demospongiae silica biosynthesis as well as its biotechnological applications (for an exhaustive and complete review see Shimizu and Morse, 2018) and animals and 3D cells models have been also developed to study the dynamics of spicule formation (Müller et al., 2006; Valisano et al., 2012; Müller et al., 2013a). In all these works, silicatein was indicated as the main molecular actor of biosilica formation. Silicatein is indeed a very peculiar protein: it has a remarkable sequence homology with the lysosomal protease family of cathepsins, but it is water-insoluble, and it has an enzymatic active site with serine in substitution to cysteine (Shimizu and Morse, 2018). Its insolubility, due to a higher number of hydrophobic regions and different post-translational modifications compared to cathepsins (Armirotti et al., 2009), determines its self-assembly properties (Murr and Morse, 2005). This evidence clearly supports the assumption of the structural role of silicatein in the formation of the axial filament of spicules as well as its being a key component of biosilica composite structure. The enzymatic active site suggests, in the meantime, the catalytic role of this protein, even if there is not a definitive agreement among scientists on the mechanism. At present time, in fact, two main hypotheses are supported in literature: silicatein as an enzyme able to catalyse the biosilica precipitation from some organosilicon compound (Cha et al., 1999; Povarova et al., 2018a; Povarova et al., 2018b) and silicatein as enzyme able to catalyse the biosilica polymerisation from orthosilicic acid (Müller et al., 2013b). Both models are apparently sustainable and silicatein can be considered one of the major contributors for the biosilica condensation in environmental conditions. It is much more difficult to understand the molecular keys of spicule growth and spicule shape control. Silicatein probably is involved in growth control and shaping, mainly in Demospongiae (Müller et al., 2009), but many other components like collagen, chitin and actin seem to play some fundamental roles (Ehrlich, 2010; Ehrlich et al., 2010; Ehrlich et al., 2016; Ehrlich et al., 2022).

A recent work (Riesgo et al., 2015) further considered the scenario concerning silicatein: a comparative analysis of transcriptomes and genomes datasets puts clearly in evidence that not all biosilica forming sponges have/express silicatein genes. Mainly Hexactinellida and Homoscleromorpha sponges do have not this protein and some silica forming Demospongiae apparently do not have silicatein (*Chondrilla nucula*). In addition, sponges that do not produce biosilica seem to express silicatein genes. Another interesting and relevant aspect is the evidence of a remarkable expression of cathepsins (specifically cathepsins B and L) in biosilica forming sponges, whose functional role is to be better clarified (Riesgo et al., 2015). In this key work the authors, on the basis of transcriptome analysis

results, hypothesized a lysosomal origin of the “silica deposition vesicle” of Demospongiae (Riesgo et al., 2015). It is evident that the biosilicification mechanisms in sponges are far to be understood, and one of the major gaps of knowledge to be filled is to improve the description of the organic content of Demospongiae spicules.

To accomplish this objective, in the present work, an integrated transcriptome and proteome analysis is proposed as a strategy. Here, for the first time, using as experimental model the species *Petrosia ficiformis*, is described the protein content of Demospongiae spicules by a proteomics analysis followed with protein sequence identification based on the transcriptome dataset matching.

## MATERIALS AND METHODS

### Protein Extraction

The proteins contained inside the silica spicules were extracted accordingly to the previously described method (Armirotti et al., 2009). Briefly (Scheme 1), 5 g of dry of *P. ficiformis* collected in

the Ligurian sea have been cut into small pieces of 1 cm<sup>3</sup> and dissolved in 500 mL of cold HNO<sub>3</sub>/H<sub>2</sub>SO<sub>4</sub> (1:4) over night. The cleaned spicules have been washed several times in distilled water until the pH was above 6, then once in 100% ethanol and finally air-dried. 1 g of clean spicules were dissolved into 100 ml of 2 M HF/8 M NH<sub>4</sub>F (pH 5) at room temperature until the complete silica solubilization. The sample was then dialysed (10,000 MWCO Da) against 5 L of Milli-Q water for 4 hours at 4°C (five changes). At the end, the insoluble organic component was recovered by centrifugation for 30 minutes at 18,000 x g at 4°C, resuspended in 3 mL of Milli-Q water and stored at -20°C until use. To check the presence of proteins in the soluble fraction, the supernatant was ten-fold concentrated and analysed using the method of Bradford assay (Bradford, 1976).

To evaluate the total protein concentration in the insoluble fraction, 0.5 mL of protein suspension were centrifuged for 5 minutes at 18,000 x g at room temperature. The supernatant was discarded, and the insoluble pellet was dissolved in 0.5 mL of 8 M urea. The protein content was evaluated by Bradford assay.

### Electrophoretic Analysis

20 µg of silica spicule protein extract were separated by SDS-PAGE according to Laemmli (Laemmli, 1970) on 10 × 8 cm vertical 12% polyacrylamide gel at a constant amperage. After electrophoresis, proteins were fixed in 40% ethanol/10% acetic acid for 60 min, washed twice with Milli-Q water, stained over night with colloidal Coomassie blue (Neuhoff et al., 1988) and destained several hours in 5% acetic acid.

### Band Cutting and Trypsin Digestion

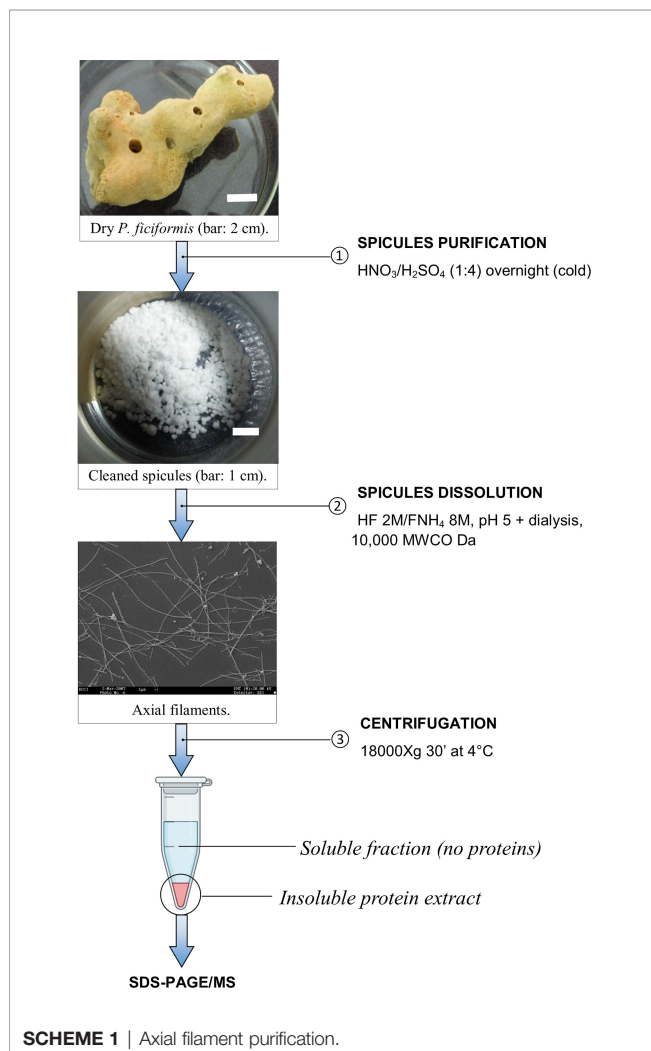
Protein bands were manually excised from the gel and digested by trypsin at 37°C overnight (Shevchenko et al., 2006). Gel pieces were destained with acetonitrile, reduced in 10 mM DTT/100 mM ammonium bicarbonate and alkylated with 100 mM iodoacetamide/100 mM ammonium bicarbonate. The peptide samples were extracted after digestion by sonicating for 10 minutes, then vacuum dried.

### Analysis of Tryptic Digests With LC-MS/MS

The LC-MS/MS method used was similar to the previously published one (Mikšik et al., 2018). The ultrahigh resolution MaXis Q-TOF (quadrupole – time of flight) mass spectrometer (Bruker Daltonics, Bremen, Germany) was coupled by nanoelectrosprayer to nano-HPLC system Proxeon Easy-nLC (Proxeon, Odense, Denmark). All controlled software was from Bruker Daltonics: HyStar (v. 3.2) micrOTOF-control (v. 3.0), ProteinScape (v. 3.0) and DataAnalysis (v. 4.0).

Three microliters of the peptide mixture were injected into trap column NS-MP-10 Biosphere C18 (particle size: 5 µm, pore size: 12 nm, length: 20 mm, inner diameter: 100 µm) and analysed by analytical column NS-AC-11-C18 Biosphere C18 column (particle size: 5 µm, pore size: 12 nm, length: 150 mm, inner diameter: 75 µm), both manufactured by NanoSeparations (Nieuwkoop, Holland).

The separation of peptides was achieved *via* a linear gradient between mobile phase A (water) and B (acetonitrile), both



containing 0.1% (v/v) formic acid. Separation was started by running the system with 5% mobile phase B, followed by a gradient elution to 30% B at 70 min. The next step was a gradient elution to 50% B in 10 min and then a gradient to 100% B in 10 min. Finally, the column was eluted with 100% B for 30 min. Equilibration between the runs was achieved by washing the column with 5% mobile phase B for 10 min. The flow rate was 0.25  $\mu\text{L}/\text{min}$ , and the column was held at ambient temperature (25°C).

On-line nano-electrospray ionization (easy nano-ESI) was used in positive mode. The ESI voltage was set to +4.5 kV, scan time: 3 Hz. Operating conditions: drying gas ( $\text{N}_2$ ): 4 L/min; drying gas temperature: 180°C; nebulizer pressure: 100 kPa. Experiments were performed by scanning from 50 to 2200 m/z. The reference ion used (internal mass lock) was a monocharged ion of  $\text{C}_{24}\text{H}_{19}\text{F}_{36}\text{N}_3\text{O}_6\text{P}_3$  (m/z 1221.9906). Mass spectra corresponding to each signal from the total ion current chromatogram were averaged, enabling an accurate molecular mass determination. All LC-MS and LC-MS/MS analyses were done in duplicate.

## Database Searching

Data were processed using ProteinScape software v. 3.0.0.446 (Bruker Daltonics, Bremen, Germany).

Proteins were identified by correlating tandem mass spectra to the putatively predicted proteins from *P. ficiformis* transcriptome, using the MASCOT searching engine v. 2.3.0 (<http://www.matrixscience.com>). Trypsin was chosen as the enzyme parameter. Extract likely coding regions and predicted candidate peptides from transcripts of *P. ficiformis* (Riesgo et al., 2014) were identified with TRANSDECODER V.5.2.0 (<https://github.com/TransDecoder/TransDecoder>), as previously described in Pozzolini et al., 2019. Three missed cleavages were allowed, and an initial peptide mass tolerance of  $\pm 15.0$  ppm was used for MS and  $\pm 0.03$  Da for MS/MS analysis. Variable modifications were set; proline and lysine were allowed to be hydroxylated, methionine oxidated, whereas asparagine and glutamine deamidated and cysteine carboxymethylated or carbamidomethylated. The monoisotopic peptide charge was set to 1+, 2+, and 3+. The Peptide Decoy option was selected during the data search process to remove false-positive results. Only significant hits were accepted (MASCOT score  $\geq 80$  for proteins and MASCOT score  $\geq 20$  for peptides), however, all peptides and proteins were additionally manually validated.

## RESULTS

### Analysis of the Proteins on Gel

The strategy of analysis of the proteins extracted from the biosilica was based on  $\text{HF}/\text{NH}_4\text{F}$  dissolution and dialysis against water (see **Scheme 1**). This approach allows the recovery of an insoluble fraction of organic material. Typically, 2 g of *P. ficiformis* clean silica spicules yielded 2 mg of total protein extract. The soluble fraction after dialysis (10,000 MWCO Da) was ten-fold concentrated and the total protein contents was assayed. No evident presence of protein was

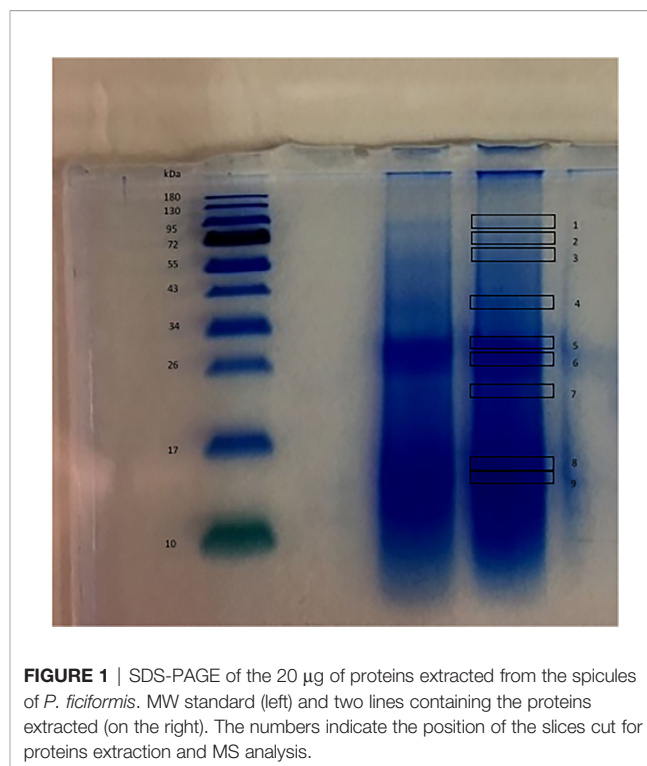
detected in the soluble fraction. The not-soluble organic residue was resuspended and 20  $\mu\text{g}$  of proteins were run on a 12% polyacrylamide gel, coloured with colloidal Coomassie blue. **Figure 1** shows the results, protein separation was not easy, and a substantial swiping of proteins is evident. The scarce solubility of the protein mixture and, probably, the presence of some silica residues could be the cause. We cut anyway the gel into 9 small slices to perform MS analysis of the protein content inside it.

### Identification and Characterisation of Proteins

The LC-MS/MS analysis raw data obtained from the organic components of *P. ficiformis* spicules were processed with the MASCOT software to search against a database consisting of transcript and predicted proteins of the above-mentioned sponge species here used as a study model.

To our knowledge, here we are showing the first case of description of the overall protein content of demosponge biosilica. In our experimental conditions, 21 proteins with a high level of confidence have been identified in their primary structure. The remarkable swiping of proteins in gel electrophoresis reflected its effects in the analytical results, where peptides of the same protein were found in different positions. In **Supplementary File 1**, the peptides and the related proteins found in the nine slices are listed. The main concentration of different proteins is present on slice 6. Some proteins are present in many or all slices (for example protein 1) while others are better distributed in the lower part of the gel.

All raw data are available on the following public repository: <https://doi.org/10.5281/zenodo.6033766>. **Table 1** summarizes



**FIGURE 1** | SDS-PAGE of the 20  $\mu\text{g}$  of proteins extracted from the spicules of *P. ficiformis*. MW standard (left) and two lanes containing the proteins extracted (on the right). The numbers indicate the position of the slices cut for proteins extraction and MS analysis.

**TABLE 1A** | List of proteins extracted from siliceous spicules of *P. ficiformis*.

Protein	peptides found	Contig	Status	AA	Predicted MW	Predicted pI
1	8	contig_2646	internal	405	45572.15	5.85
2	4	contig_3602	5'-partial	463	50256.01	5.39
3	3	contig_5736	internal	254	28039.31	5.22
4	2	contig_7487	internal	436	48710.29	4.96
5	5	contig_3356	complete	340	37361.94	6.12
6	4	contig_6478	complete	334	37069.50	5.16
7	3	contig_910	complete	239	27596.48	4.65
8	2	contig_457	5'-partial	179	20405.85	4.98
9	1	contig_5079	3'-partial	155	17868.96	5.93
10	1	contig_6226	3'-partial	155	17960.19	5.93
11	4	contig_3070	5'-partial	146	16175.06	6.17
12	4	contig_313	5'-partial	338	37003.89	5.37
13	4	contig_183	complete	152	17240.88	7.10
14	4	contig_8334	5'-partial	720	not calculated	n.c.
15	4	contig_1552	complete	340	38075.11	5.49
16	4	contig_17524	3'-partial	171	19212.98	6.42
17	1	contig_9379	internal	120	12431.88	4.28
18	2	contig_2754	complete	362	40277.86	5.69
19	2	contig_7352	5'-partial	111	12782.54	7.77
20	1	contig_2762	5'-partial	418	45731.84	5.42
21	4	contig_3044	complete	165	17728.46	4.46

**TABLE 1B** | Main conserved domains.

Protein	Main domains/regions found	Signal peptide	Transmembran domains	Similar protein	Lisosome related
1	Pfam sulfatase	Y	N	Arylsulfatase A-like (E-value 1e-179). Acc. Num. XP_003387234.3	Y
2	SCOP d1iexa1 (E-value 2.00e-08), Pfam Glyco_hydro_3_C (E-value 1.4e-40), Fibronectin type III-like dom. (E-value 0.000822)	N	N	Probable beta-D-xylosidase 2 (E-value 1e-156), fam. Glycosyl hydrolase. Acc.Num. XP_021231341.1	Y
3	Pfam Glyco_hydro_3 (E-value 4.1e-38)	N	N	Probable beta-D-xylosidase 6 (E-value 3e-126), fam. Glycosyl hydrolase. Acc. Num. XP_028393522.1	Y
4	Pfam Glycohydro_20b2 (E-value 1.1e-17), Pfam Glyco_hydro_20 (E-value 3.1e-77)	N	N	Beta-hexosaminidase subunit alpha-like isoform X4 (E-value 0.0). Acc. Num. XP_019855835.1	Y
5	Inhibitor_I29 (E-value 7.58e-20), Pept_C1 (E-value 2.59e-93)	Y	N	Silicatein (E-value 0.0). Acc. Num. Q6YD92.1	Not known
6	Inhibitor_I29 (E-value 1.47e-17), Pept_C1 (E-value 1.59e-111)	Y	N	Cathepsin L1-like (E-value 1e-152). Acc.Num. XP_003383726.1	Y
7	PTPc_DSPc (E-value 1.84)	Y	N	Tyrosine phosphatase (E-value 1e-26). Acc. Num. KYR01243.1	Y
8	No domains predicted	N	N	UDP-N-acetylglucosamine-LPS N-acetylglucosamine transferase (E-value 2.4). Acc. Num. KYR01243.1	
9	Inhibitor_I29 (E-value 4e-19), Pfam Peptidase_C1 (E-value 2.7e-14)	Y	N	Cathepsin L1-like (E-value 2e-33). Acc. Num. XP_003385986.1	Y
10	Inhibitor_I29 (E-value 1.6e-18), Pfam Peptidase_C1 (E-value 2.8e-13)	Y	N	Cathepsin L1-like (E-value 5e-34). Acc. Num. XP_003385986.1	Y
11	No domains predicted	N	N	Cation-dependent mannose-6-phosphate receptor (E-value 0,75). Acc. Num. XP_011403525.2	Y
12	Pfam: Propeptide_C1 (E-value 2.4e-14), Pept_C1 (E-value 2.34e-91)	Y	N	Cathepsin B-like ((E-value 6e-166). Acc. Num. XP_003388842.1	Y
13	MD-2-related lipid-recognition (ML) (E-value 0.0000946)	N	N	NPC2-like protein (E-value 5e-06) Acc. Num. KOX75392.1	Y
14	Three PbH1 (E-values 4.11e+03, 2.02e+03, 2.46e+02)	N	N	Possible polysaccharide lyase.	Not known
15	Pfam Metallophos (E-value 1.2e-18)	N	Y	Tartrate-resistant acid phosphatase type 5-like isoform X3 (E-value 8e-153). Acc. Num. XP_019850709.1	Y
16	Pfam DUF547 (E-value 1.1e-06)	Y	N	Lipoprotein precursor (E-value 5e-15). Acc. Num. ARU54835.1	Not known
17	Pfam Aps (E-value 9.5e-26)	N	N	Lysosomal aspartic protease-like (E-value 1e-33), Cathepsin-D (E-value 8e-33)	Y

(Continued)

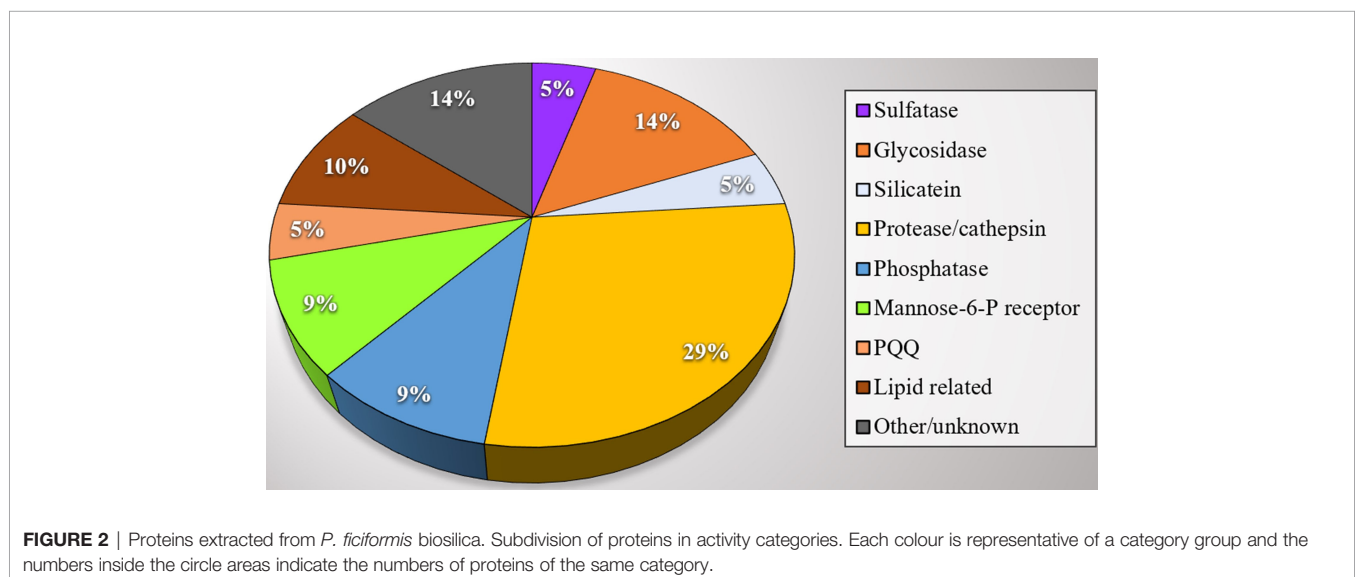
TABLE 1B | Continued

Protein	Main domains/regions found	Signal peptide	Transmembran domains	Similar protein	Lysosome related
18	Inhibitor_I29 (E-value 1.68e-12), Pept_C1 (E-value 2.73e-92)	N	N	Digestive cysteine proteinase 1-like (E-value 1e-153)	Y
19	No domains predicted	N	N	Mannose 6-phosphate receptor domain (Phyre2)	Y
20	Three PQQ (E-values 410, 41.9, 23.7)	Y	N	Pyrrolo-quinoline quinone (E-value 7e-05)	Not known
21	MD-2-related lipid-recognition (ML) (E-value 5.46e-19)	Y	N	Putative phosphatidylglycerol/phosphatidylinositol transfer protein (E-value 3e-34)	Y

Proteins extracted from the 9 gel slices. **A:** number of peptides identified, the corresponding contig and relative status, number of Amino acids, predicted MW and PI. More details are reported in **Supplementary Files 1, 3. B:** main conserved domains (see **Figure 3**), presence of signal peptide and/or transmembrane domains, similar proteins, and correlation with lysosome environment. More details are reported in **Supplementary File 3**.

the structural features of the identified proteins and their theoretical functions based on the predicted primary structure and of the analysis of conserved domains. Spicule protein content in our findings can be divided into functional categories, as shown in **Figure 2**. Most proteins seem remarkably related to the lysosome environment (see **Table 1**) and only a few are of unknown origin. In **Figure 3** the specific domains of each protein characterized in this work are shown. A detailed overview of the results includes the presence of *P. ficiformis* silicatein beta, as expected (protein 5, **Table 1**). Silicatein peptides were detected from bands 4 to 9 with a typical swiping. With similar swiping behaviour, five different proteases of the cathepsin family (proteins 6, 9, 10, 12, 17) have been identified. The larger part of the other proteins in the list has remarkable similarity with lysosome-related polypeptides. Protein 1 shows strong homology with the aryl-sulphatase family enzymes, as in the predicted primary structure are present all the amino acids of the active site included the typical Cys residue normally modified in C-alpha-formyl-glycine by formyl-generating enzymes of rough endoplasmic reticulum. These enzymes are known to catalyse the cleavage of sulphate esters mainly in glycolipids and glycosaminoglycans inside lysosomes (Thompson and Daniel, 1988). Proteins 2, 3, and 4 have their

primary structure remarkably homologs to glycosyl hydrolase family 3 (protein 2 and 3), and beta-hexosaminidase group (protein 4). All these proteins are involved in the catabolism of glycosphingolipids inside lysosomes (Ryckman et al., 2020). In mammals, it is known that glycosyl hydrolases of family 3 are involved in the transxylosylation of cholesterol in lysosomes. Beta-hexosaminidases are involved in the hydrolysis of terminal N-acetyl-D-hexosamine residues in N-acetyl-β-D-hexosaminides and it is typically expressed inside lysosomes for glycosphingolipids catabolism. In the same “group” of proteins related to lipid catabolism it is included protein 13, whose primary sequence is remarkably related to cholesterol transporter from lysosomes as well as protein 21 whose lipid recognition domain is typically present in phosphatidylinositol/phosphatidylglycerol transfer protein, fundamentally found inside lysosomes. The 21 proteins list also includes proteins with possible mannose-6P receptor domain, characteristic of protein trafficking to lysosomes (proteins 11 and 19), in particular interacting with cathepsins and hexosaminidases (Staudt et al., 2017). Tyrosine phosphatases and tartrate-resistant phosphatases (proteins both present in the lysosome environment) are also represented in this very complex pattern of biomolecules found inside biosilica. The list includes



one protein with a domain corresponding to pyrrole quinoline quinone dehydrogenase. This protein is not typical of the lysosome environment. Finally, three unknown proteins are also represented.

## DISCUSSION

The biomineralization process in Porifera still has several unsolved questions. Most of the scientists studying biosilicification in demosponges attribute to silicatein a key role in the skeleton forming process, (see the recent review, Shimizu and Morse, 2018). Indeed, silicatein is very abundant inside spicules and its distribution is intimately mixed with the inorganic part to form a composite (Müller et al., 2013; Shimizu and Morse, 2018). Nevertheless, at present, this peculiar protein, with its proposed double role (enzymatic and structural), in our opinion is not enough to fully explain all the aspects of the biosilicification mechanism in these animals. In biological controlled biomineralization, the molecular actors are very numerous, involving proteins and other biomacromolecules (polyamines for example), whose role in the process is often very difficult to identify (Wysokowski et al., 2018). Proteomic analysis in these cases give a relevant contribution to identify the various component of an organic compound and in the past, it has already been used to describe the post-translational modification of silicatein (Armirotti et al., 2009). In the present study we have improved the strategy looking again inside spicules using an integrated approach based on proteome and transcriptome analysis. Recently, similar methodologies were used to describe the organic matrix content of diatoms and corals (Kotzsch et al., 2016; Peled et al., 2020).

Using this technique, we have found together with silicatein a list of proteins until now never described before inside biosilica. The *P. ficiformis* transcriptome availability (Riesgo et al., 2014) allowed in the present work a fine identification of proteins inside spicules (**Figure 2** and **Table 1**) but, for many of them, it is not easy to attempt a possible explanation of their role in the process. Previous scientific publications demonstrate that the spicule formation in demosponges begins inside cells, but it continues outside, and the process pass through the production of biosilica building units inside cells successively secreted outside (Wang et al., 2011). A last century study (Lévi, 1963) and a more recent description of spicule formation by Schröder and co-workers (Schröder et al., 2007) show the formation of specific intracellular vesicles in which biosilica is precipitated. In accordance with what is described in many other biomineralization processes, the building blocks are organized intracellularly in appropriate compartments where the chemical conditions for mineral precipitation are created and maintained. The ultrastructural analysis reported in the work of Schröder and co-workers gives some explanation of the process: silica is released in extracellular granules outside the sclerocyte; these granules are rich in silica, and they are named silicasomes. Morphological analysis showed that silicasomes positively react with antibodies against silicatein, and this reaction allowed to follow the intra- and extracellular evolution of these organelles (Schröder et al., 2007). This evidence suggests a subcellular process involving some systems

related to endosome/exosome or microvesicles pathways. Furthermore, it is known that the acidic environment favours biosilica precipitation (Shimizu et al., 2001) and, for this reason, also lysosome or lysosome-related organelles may be involved in the formation of silicasomes. In this conceptual direction it is also placed the co-expression of silicatein and cathepsins recently described in sponge tissues by Riesgo and co-authors, suggesting some specific roles of these proteins in the biomineralisation process as well as clearly remarking the hypothesis of a silica deposition environment of lysosomal origin (Riesgo et al., 2015). In this study Riesgo and co-authors made an accurate analysis of the evolution of cathepsin genes among metazoan and many of them have been described in sponges. Specifically, in *P. ficiformis* transcriptome they documented the expression of Cathepsin B, L, and D as well as 4 different silicateins (Riesgo et al., 2015). This information derives from comparative transcriptomic analysis of sponges, without any further insights into protein localisation. Our findings demonstrate, for the first time, the presence of cathepsins and silicatein inside the biosilica, fully confirming the lysosome hypothesis. Additionally, in our research results many other proteins are for the first time identified inside *P. ficiformis* spicules after inorganic silica removal, almost half of them is provided with a signal peptide and shows a strong similarity with proteins normally found inside lysosomes (see **Table 1**). The scenario is however very complex: from one hand the common lysosomal origin of the majority of proteins listed in our work is something coherent and easy to discuss on the basis of the morphological evidence published (Schröder et al., 2007) and of the evolutionary study cited (Riesgo et al., 2015); on the other hand, it becomes very difficult to attribute a specific role in the process for many of the proteins here identified.

The first aspect emerging from the analysis of biosilica protein content shown in **Table 1** of our results is the demonstration of presence of some isoforms of cathepsin family together with silicateins, data strongly congruent with the previously published hypothesis of Riesgo (Riesgo et al., 2015). Riesgo and co-authors already suggested a possible role of cathepsins in the biosilicification process (Riesgo et al., 2015). In particular, the fact that one silicatein cited by Riesgo has cysteine inside the active site is suggestive of a more complex scenario where some cathepsins can play some role (Riesgo et al., 2015). In addition, it also known that mutants of L cathepsin produced in recombinant systems confirm the existence of structural issues other than active sites in the biosilica biosynthesis in silicateins and cathepsins (Fairhead et al., 2008). On these bases, the changing of the active site from Cys to Ser is not the only molecular key of the mechanism, but also other regions of the protein can have a role in the catalysis and/or in the biosilica organisation and/or in other proteins assembling (Fairhead et al., 2008). Despite all these considerations, definitively it is not easy to define the exact role of various cathepsins in the biosilica precipitation environment: one additional possibility we suggest is that they can be involved in the specific activation of other proteins relevant in the process (*i.e.*, cleaving signal peptides and/or regulatory peptides) but more studies must be performed to better understand if these roles are related only to catalysis or also to structural organisation. Only future experimental

demonstrations based on selective enzymatic inhibition or gene silencing approach in cellular models can give a final answer.

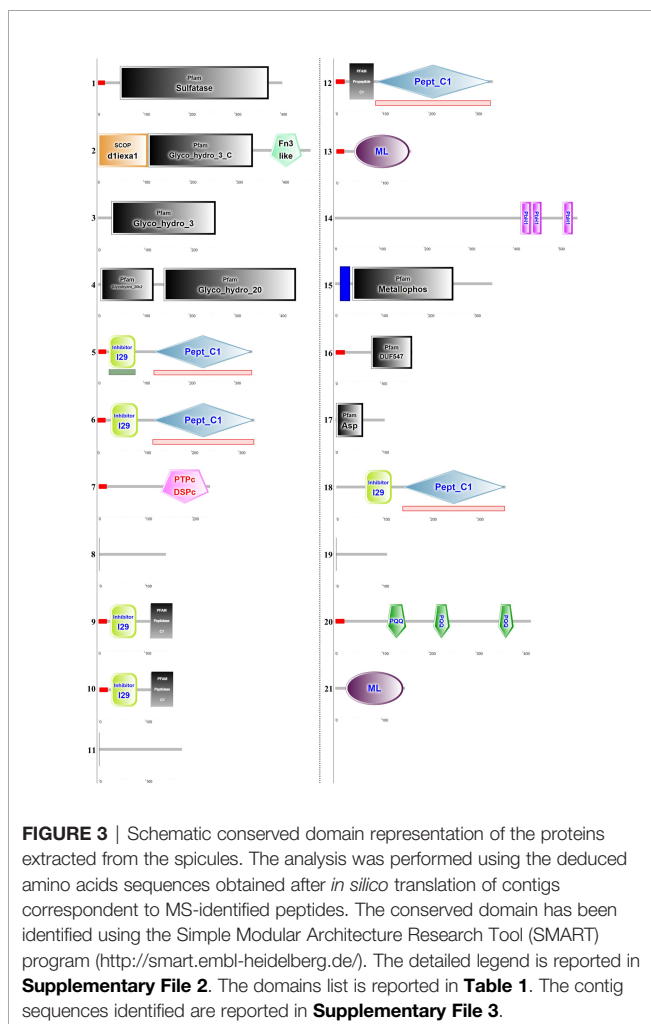
In **Figure 3** and in **Supplementary File 2** are listed some details of domain and sequence homology also of the other proteins found and, in the results, we have underlined the enzymatic roles of the homologous polypeptides as described in other organisms. But, if it is difficult the possible explanation of cathepsins and silicateins roles, the discussion of other proteins here found in the biosilica matrix only on the basis of their structural similarity with other well-known proteins of lysosomal origin is much more speculative. A further interesting aspect is that many of these proteins have well defined enzymatic domains (see **Figure 3**), differently from other situations (corals and diatoms for example), where a considerable numbers of proteins with structural functions and apparently lacking catalytic domains have been found (Ramos-Silva et al., 2013; Kotsch et al., 2016). In our research results we conversely identified aryl sulphatase, glycosyl hydrolase, beta-hexosaminidase, all typical proteins found inside lysosomes. Also, proteins involved in the lysosome cholesterol transport have been found (see **Table 1A** and **Figure 2**). Only three

proteins are of unknown origin, (with some low similarities to acetylglucosamine transferase, polysaccharide lyase and one lipoprotein).

The presence of enzymes related to glycosaminoglycans (GAGs) and polysaccharides catabolism inside biosilica is indeed intriguing. The sugars, the polyol and the GAGs interaction with silicon recently suggested by Nielsen (Nielsen, 2014) and the regulation of connective tissue formation in mammals influenced by silicon is suggestive of some biochemical mechanism involving silicon and GAGs and other polysaccharides in mammals (Götz et al., 2019). The presence of these enzymes inside of silica sponge spicules suggest a possible sugars-silicon interaction originally involved in biomineralization. A future explanation of their role can give important answers not only in biosilicification process in sponges but also in cartilage and bone formation in mammals and humans. The results here shown undoubtedly confirm the lysosome hypothesis since the list of proteins here found are in their majority clearly related to lysosomes, but at the same time opens the door to many unanswered questions, specifically on the metabolic management of silicon inside cells. The silicon uptake in sponges is under the control of specific transporters (Schröder et al., 2004; Maldonado et al., 2020), but the intracellular destiny of silicon is not clear. The hypothesis of one organosilicon compound already emerged from the beginning of studies of silicatein (see Shimizu et al., 1998) but, at the same time no specific ideas have already been confirmed. Bio-organic chemistry works suggested the general possibility to have silica derivatives with organic molecules of biological relevance (Kinrade et al., 1999; Vis et al., 2020). The presence of many enzymes related to sugar metabolism could be suggestive of silicon-organic compounds specifically involved in this process, while cathepsins and silicateins can play a role both in the enzymatic control of biosilica precipitation and in the structural organisation of spicules.

Much more difficult is to give some functional hypothesis of other proteins. Another finding is a protein with pyrrolo-quinoline quinone (PQQ) dehydrogenase domain. This family of proteins are normally present in prokaryotes but recently they have been described also in Eukaryotic, involved in sugars oxidoreductases process (Matsumura et al., 2014).

In this scenario, the apparent absence of silintaphins (Wiens et al., 2009) and galectins (Schröder et al., 2006) is surprising. In the first case, also inside the template transcriptome (Riesgo et al., 2014) we have not found silintaphins. We cannot currently confirm if this is due to incompleteness in transcriptome database used in this study or to some specific genetic differences that occurred in this sponge species. Different considerations should be made regarding galectins. In this second case, in the transcriptome database used, the presence of galectin transcript is confirmed, but any traces of these proteins in the proteomic analysis was not found, despite the presence of galectins in the biosilica matrix was proposed using specific antibodies in a previous work on *S. domuncula* (Schröder et al., 2006). In our experimental conditions, we performed a





strong acid spicules cleaning before silica dissolution. Differently, in the study of Schröder and co-workers the extraction procedure was completely different and milder (Schröder et al., 2006) and maybe some proteins involved in the biosilica building not intimately mixed inside biosilica in our approach can be missed. The methodological approach in the extraction procedure without doubt can affect the results. Here we have used this very strong cleaning process (cold HNO<sub>3</sub>/H<sub>2</sub>SO<sub>4</sub>) of spicules in order to avoid contaminations. MS analysis, in fact, is highly sensitive and it is not easy to evaluate proteins at the quantitative level. This sensitivity on the other hand allows to identify many proteins not found before with different analytical approaches. Here we decide to focus our attention to those proteins very intimately mixed with the inorganic silica lattice.

## CONCLUSIONS

Biomineralization in sponges is a process not completely explained. In this work, using a strong spicules cleaning procedure, we have extracted the organic proteinaceous material embedded inside biosilica spicules from the marine sponge *P. ficiformis* and analysed by MS analysis. The results obtained confirmed the hypothesis of lysosome origin of silicasomes, due to the identification of various cathepsins together with silicateins and surprisingly we found many proteins similar to lysosome enzymes, several of them related to polysaccharide and sugar metabolism. Taken together, these results open a new perspective in the study of biosilica forming in demosponges, suggesting a more complex scenario where sugars and maybe lipids can have key roles.

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## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: <https://doi.org/10.5281/zenodo.6033766>.

## AUTHOR CONTRIBUTIONS

Conceptualization: MP and MG. Methodology: MP, IM, SG, CO, ET, and MG. Investigation: MP, IM, SG, CO, and ET. Writing: MP and MG. Review and editing: MG and ET. Supervision: MP and MG. Funding acquisition: MP, IM, and MG. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.850653/full#supplementary-material>

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