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Original article

First evidence of ovothiol biosynthesis in marine diatoms

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ABSTRACT

Ovothiols are histidine-derived thiols that are receiving a great interest for their biological activities in human model systems. Thanks to the position of the thiol group on the imidazole ring of histidine, these compounds exhibit unusual antioxidant properties. They have been revealing a very promising pharmacological potential due to their anti-proliferative and anti-inflammatory properties, as well as anti-fibrotic activities not always related to their antioxidant power. Ovothiols occur in three differentially methylated forms (A, B and C), isolated from ovary, eggs and biological fluids of many marine invertebrates, mollusks, microalgae, and pathogenic protozoa. These molecules are synthesized by two enzymes: the sulfoxide synthase OvoA and the sulfoxide lyase OvoB. OvoA catalyzes the insertion of the sulfur atom of cysteine on the imidazole ring of histidine, leading to the formation of a sulfoxide intermediate. This is then cleaved by OvoB, giving 5-thiohistidine, finally methylated on the imidazole ring thanks to the methyltransferase domain of OvoA. Recent studies have shown that OvoA homologs are encoded in a wide variety of genomes suggesting that ovothiol biosynthesis is much more widespread in nature than initially thought. Here we have investigated the OvoA occurrence in diatoms, one of the most abundant group of microalgae, dominating marine and freshwater environments. They are considered a very good model system for both biology/photophysiology studies and for biotechnological applications. We have performed comparative sequence and phylogenetic analyses of OvoA from diatoms, highlighting a high degree of conservation of the canonical domain architecture in the analyzed species, as well as a clear clustering of OvoA in the two different morphological groups, i.e. centric and pennate diatoms. The *in silico* analyses have also revealed that *OvoA* gene expression is modulated by growth conditions. More importantly, we have characterized the thiol fraction from cultures of the coastal centric diatom *Skeletonema marinoi*, providing the first evidence of ovothiol B biosynthesis in diatoms.

1. Introduction

Ovothiols are 5(*N*π)-methylated thiohistidines, initially isolated from the eggs and biological fluids of many marine invertebrates and more recently identified in protozoa and microalgae [1]. These compounds are characterized by uncommon antioxidant properties due to the position of the thiol group on carbon 5 of the imidazole ring of histidine, which confers to this group an extremely low basicity and determines a very low pK_a value (1.4) and a much more positive redox potential (−0.09 V) compared to other intracellular thiols, such as glutathione (pK_a = 8.75, redox potential = −0.26 V) [2–4]. Ovothiols have recently shown anti-proliferative activities in human hepatocarcinoma and chronic leukemia cell lines, by inhibiting human γ-glutamyl transpeptidase [5–7], as well as they act as anti-inflammatory agents both in endothelial cells from women affected by gestational diabetes and in *in vivo* murine models of liver fibrosis [8–10].

Three forms of ovothiols, A, B and C, have been characterized, differing in the extent of methylation at the α-amino group. Ovothiol A (1) lacks this additional methyl group and is the most widespread form in nature, being produced by a variety of invertebrates, such as sea urchins, starfish, holothurians, mollusks and marine worms [1] and recently also identified in fish eye lenses [11]. Ovothiol B (2), with one methyl group, was so far only found in the ovary of the scallop *Chlamys hastata*, whereas ovothiol C (3), with two methyl groups, was isolated from the eggs of the sea urchins *Sphaerechinus granularis* and *Strongylocentrotus purpuratus* [1].

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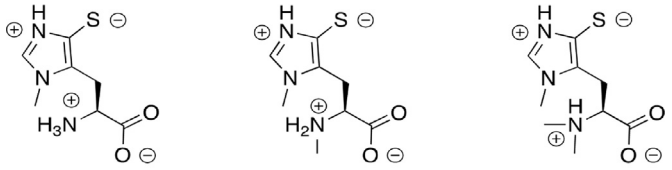

(1) (2) (3)

While the effect of the different methylation state on the physiological function of ovothiols is still not clear, their biological roles have been studied in different organisms, revealing to be quite wide and diverse in nature. Indeed, they protect sea urchin eggs from the oxidative burst at fertilization and developing embryos from environmental cues [12,13], while in marine worms they might act as pheromones in sexual reproduction [14,15]. In the microbial world, they protect pathogenic parasites from the oxidative stress produced by macrophages during infection [4,16]. Also a role in protection from light-dependent stress in microalgae was suggested [17,18].

The ovothiol biosynthetic pathway has been almost fully characterized. The first step of ovothiol biosynthesis, i.e. the insertion of the sulfur atom of cysteine into the C5 position of the imidazole ring of histidine, is catalyzed by a sulfoxide synthase (OvoA), first characterized from the bacterium *Erwinia tasmaniensis* and the protozoon *Trypanosoma cruzi* [19]. OvoA is an iron-dependent bifunctional enzyme with a N-terminal sulfoxide synthase and a C-terminal S-adenosyl methionine (SAM)-dependent methyltransferase (MT) [19]. The sulfoxide synthase activity, performed by a DinB superfamily domain and a formylglycine-generating (FGE)-sulfatase domain, is responsible for the formation of the sulfoxide intermediate, which is then cleaved by an unspecific pyridoxal 5′-phosphate-dependent β-lyase (OvoB) leading to 5-thiohistidine (5-thio) [20]. Finally, a methyl group is added on the N_{π} of the imidazole ring of the 5-thio by the catalytic action of the MT domain of OvoA [20], leading to the formation of ovothiol A. The MT responsible for the addition of the methyl groups on the lateral $\alpha-$amino group, thus forming ovothiol B and C is still unknown. The identification and characterization of OvoA pointed out that homologs of this enzyme are encoded in a wide range of genomes, from proteobacteria to animalia, revealing that ovothiol biosynthesis is much more widespread in nature than initially thought [21]. Up to now, the evolutionary history of OvoA has only been investigated in metazoans, revealing a high degree of conservation from Porifera, Placozoa, Cnidaria (Anthozoa) to protostomes (Annelida and Mollusca) and deuterostomes, while several gene loss events occurred especially in Ecdysozoa and vertebrates, after the split between Chondrichthyes and Teleostomi, resulting in the lack of OvoA orthologs in all extant bony fish and tetrapods [13,22]. While OvoA homologs have been identified in diatoms [19,22], ovothiol molecules have not been detected so far. Diatoms are one of the most abundant groups of microalgae, inhabiting all aquatic environments and contributing to one-fifth of the photosynthesis on earth [23,24]. They possess a much more complex evolutionary history compared to land plants, because diatom evolution involved the occurrence of two endosymbiotic events: the first one, between a eukaryotic heterotroph host and a cyanobacterium, giving rise to the progenitor of land plants, red and green algae; and a second one, between a different heterotroph and a red alga, forming the progenitor of diatoms, brown algae and oomycetes [25]. Moreover, gene transfer from bacteria and viruses contributed to the unique combination of genes and pathways in diatoms, presumably allowing them to adapt to new ecological niches [26,27]. A fascinating feature of diatoms is their silica wall (frustule), which can assume the most diverse forms, accounting for the classification of these microalgae in two main groups: centric, evolutionary older, and pennate, younger, differing in the symmetry of the frustule [28,29]. Interestingly, the high growth rate, the ability to adapt to different environmental conditions and the possibility to modulate their growth and physiology to optimize their biomass yield and metabolites production, make diatoms very promising "biofactories" for biotechnological applications [30–32]. Indeed, the use of diatoms as a rich source for bioactive compounds, like carotenoids, vitamins, antioxidants and polyunsaturated fatty acids, and the manipulation of their growth conditions, especially light, has been greatly expanding [33–37].

The aim of this study was to investigate the occurrence of the ovothiol biosynthesis in diatoms by different approaches. We have inferred the distribution of OvoA homologs in diatoms by comparative sequence analyses and neighbor-joining phylogeny of the protein sequences retrieved by public databases. We have also predicted the tertiary structure of OvoA enzyme from the coastal centric diatom *Skeletonema marinoi*, promising model organism for biotechnological investigations [35,36]. Finally, we have characterized the thiol fraction from *S. marinoi* cultures, identifying ovothiol B as the 5-thiohistidine produced by these microalgal cells.

2. Materials and methods

2.1. Culture conditions and samples preparation

The coastal centric diatom *Skeletonema marinoi* was used as model species due to the high growth rate [38,39], its use as feed in aquaculture [40–43] and other potential biotechnological applications [35,36]. Yet, a lot of information on the biology and photophysiology of this species is available [32,44]. The strain (CCMP 2092) of *S. marinoi* used in this work was collected from the surface waters of the northern Adriatic Sea, where this diatom provides the major contributor to the late winter blooms [45]. While the sequencing of *S. marinoi* genome is ongoing, the transcriptome is already available [46].

S. marinoi cultures were carried out in 4.5 L glass flasks with air bubbling at 20 °C, containing seawater previously pre-filtered through a 0.7 mm GF/F glass-fiber filter (WhatmanTM, Whatman International Ltd, Maidstone, UK), autoclaved and enriched with F/2 medium nutrients [47]. Light was provided at a moderate non stressful sinusoidal light, peaking at 150 μmol photons s^{-1} m^{-2} after 6 h from dawn (low sinusoidal light 150, LSL150), with a 12:12 light:dark photoperiod, by a custom-built LED illumination system which allows to modulate light intensity and spectral composition (Patent number: EP 13196793.7) [48]. The spectral composition of the light was kept constant (blue:-green:red = 50:40:10). Light intensity was measured inside each flask by using a laboratory PAR4π sensor (QSL 2101, Biospherical Instruments Inc., San Diego, CA, USA). Cultures were performed in triplicate and, from each flask, about 100 × 10^6 cells in the exponential growth phase were collected after 6 h from dawn (i.e., at the maximal peak of light intensity). Samples were then centrifuged at 3600 rpm speed, 4 °C for 15 min using an Eppendorf Centrifuge 5810 R, Rotor A-4-62. Pellets were lyophilized using an Edwards lyophilizer. The dried pellets were weighted (flask 1 = 8.19 mg; flask 2 = 7.96 mg; flask 3 = 7.26 mg) and analyzed by HPLC/HR-ESI-MS.

2.2. Cell density

Two mL sub-samples from each flask were collected in duplicate and fixed with Lugol's iodine solution (1.5% v/v). One mL of this solution was used to fill a Sedgewick Rafter counting cell chamber and cell counts were performed by using a Zeiss Axioskop 2 Plus light microscope (Carl Zeiss, Göttingen, Germany).

2.3. Ovothiol identification and characterization

Freeze-dried cells were rehydrated by adding deionized water (2.5 μl per mg dry weight). 100 μL extraction buffer (acetonitrile (ACN):0.75 M perchloric acid, 1:2) were added to 25 mg of cell suspension. The sample was vortexed (3 × 20 s) and centrifuged for 5 min at 14000 rpm to remove cellular debris. 100 μL cleared lysate was

neutralized by addition of 15 μL of 2 M potassium carbonate. The mixture was centrifuged for 2 min at 14000 rpm to remove insoluble potassium perchlorate. 100 μL of the supernatant were basified with 10 μL lithium carbonate (50 mM, pH 10). To reduce all thiols in the extract, 3 μL of 100 mM DTT were added and the reaction was incubated for 5 min before 25 μL of a 20 mM DMSO solution of 4-bromomethyl-7-methoxycoumarin (BMC) was added. After 30 min the mixture was acidified by the addition of 100 μL 1% trifluoroacetic acid (TFA) in water. Samples were vortexed for 10 s to remove CO_2 and then centrifuged for 10 min to remove excess BMC. Samples were analyzed by reversed phase HPLC using a Gemini-NX C18 (5 μm, 250 × 4.6 mm) column on a Shimadzu Prominence series apparatus. Thiol-BMC conjugates were detected with a diode-array detector at 330 nm. The HPLC method was as follows: solvent A is H_2O/ACN/TFA 100:1:0.1, solvent B is ACN/TFA 100:0.085. The flow rate was 1 ml/min. Linear gradients were used for separation (0 min, 2% B; 1.5 min, 2% B; 22.5 min, 70% B; 24.5 min, 95% B; 29.5 min, 95% B; in 31.5 min, 2% B; 34.5 min, 2% B). Peak areas were integrated and converted to concentrations using glutathione-BMC for calibration. Isolated ovothiol-BMC adducts were characterized by HR-ESI-MS. Ovothiol B identification was confirmed by co-elution with an authentic standard (Seebeck, unpublished data). Ovothiol B and glutathione contents were calculated as wt% on freeze dried cells.

2.4. In silico analysis of the 5-histidylcysteine sulfoxide synthase (OvoA) in diatoms

The *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* OvoA protein sequences were downloaded from the NCBI protein database. The *Skeletonema marinoi* (strain CCMP2092) OvoA (*Sm*OvoA) protein sequences were downloaded from the MMETSP (Marine Microbial Eukaryote Transcriptome Sequencing Project) website (www.imicrobe.us/#/search/MMETSP) (trascriptome ID: MMETSP1039; OvoA sequences IDs: 2388, 4090). The sequences of *Paracentrotus lividus* (AMM72581.1) and *Strongylocentrotus purpuratus* (XP_011674521.1) OvoA were previously identified [13]. The sequences were aligned with ClustalX (version 2.1) and edited using GeneDoc software. The analysis of domains was performed using InterPro database. MMETSP and NCBI databases were searched for OvoA occurrence in diatoms, resulting in Supplementary Tables S1 and S2 Transcriptomic data available in GEO database on the pennate diatom *P. tricornutum* [49,50] were also analyzed, through GEO2R web tool (GSE42039, GSE55959), for *OvoA* gene expression.

2.5. SmOvoA structural modelling

*Sm*OvoA tertiary structure was predicted through structural modelling using SWISS-MODEL server (http://swissmodel.expasy.org/) and the crystal structure of the Ergothioneine biosynthetic enzyme EgtB from *Chloracidobacterium thermophilum* as template (PDB code 6QKJ) [51]. UCSF ChimeraX (http://www.cgl.ucsf.edu/chimera/) was used to visualize the EgtB crystal structure and *Sm*OvoA model and obtain figures.

2.6. Phylogenetic analysis

The phylogenetic tree of OvoA homologs from diatoms was inferred using the Neighbor-Joining method [52]. The evolutionary distances were computed using the JTT matrix-based method [53] and are in the units of the number of amino acid substitutions per site. The analysis involved 50 amino acid sequences, downloaded from the NCBI protein database and MMETSP website (all accession numbers are reported in Fig. 1 and Supplementary Table S1). Forty-five of these sequences were from diatom species, whereas 5 were from Heterokonta and Chlorophyta phyla, used as outgroups. All positions containing gaps and missing data were removed, and the final dataset was composed by a total of 239 positions. Evolutionary analyses were conducted in MEGA7 [54].

3. Results

3.1. Occurrence and distribution of ovoA in diatoms

The occurrence of ovoA homologs in diatoms was disclosed and analyzed through comparative sequence analyses. Available diatom genomes, i.e. *Fragilariopsis cylindrus*, *Phaeodactylum tricornutum*, *Pseudo-nitzschia multiseries*, *Thalassiosira oceanica* and *Thalassiosira pseudonana*, were searched for the *OvoA* occurrence. When the genome was not available, transcriptomic data were used. The overall results revealed the presence of the ovoA transcript in 45 diatom species on a total of 72 screened species. These included the centric diatoms *Skeletonema marinoi*, *Thalassiosira pseudonana* and *Leptocylindrus danicus*, and pennate diatoms *Fragiliaropsis cylindrus*, *Pseudo-nitschia delidatissima* and *Phaeodactylum tricornutum* (Supplementary Table S1). No ovoA was found in 27 diatom species for which the transcriptomes were available, such as in the centric diatoms *Chaetoceros sp.*, *Skeletonema japonicum* and *Thalassiosira minuscola*, and the pennates *Asterionellopsis glacialis*, *Amphiprora paludosa* and *Craspedostauros australis*. In some species ovoA was not found in all strains, such as in *Minutocellus polymorphus*, in which ovoA was found in the strains RCC2270 and NH13, but not in the strain CCMP3303. Also in *S. marinoi*, ovoA was present in the strains skelA and CCMP2092 (used in this work), whereas it was absent in the strains FE60, SM1012Hels-07, UNC1201 and SM1012Den-03. Moreover, in some species, such as *Leptocylindrus danicus* (strain B650), *Minutocellus polymorphus* (strain NH13), and *S. marinoi* (strain CCMP2092) two ovoA transcripts were found (Supplementary Table S1).

The OvoA protein sequences retrieved from the available diatom genomes and transcriptomes, representing 28 genera, were used to perform a multiple sequence alignment and reconstruct OvoA phylogeny in diatoms by neighbor-joining (NJ) algorithm. Sequences from Heterokonta and Chlorophyta phyla were used as outgroups. The results showed that OvoA homologs cluster in two different clades reflecting, to some extent, the diatom evolution divergence in centric and pennate species (Fig. 1).

3.2. Transcriptional regulation of ovoA

The analysis of different transcriptomes obtained under different growth conditions allowed to highlight that, in some species, the presence of the ovoA transcript depends on nutrients availability and light intensity (Supplementary Table S2). In the centric diatom *Thalassiosira weissflogii*, for example, the ovoA transcript was found under iron (4-10 nmol/L), silicate (10.6 μmol/kg) or nitrate (0–88.2 μM) limitation, while it was absent in replete conditions (iron: 400 nmol/L; silicate 106 μmol/kg; nitrate 882 μM). In *S. marinoi* (strain Skel A), high concentration of phosphate and low light conditions induced the expression of *ovoA*. Yet, *Extubocellulus spinifer* exhibited *ovoA* expression when cultured under low light condition and in presence of nocodazole (Supplementary Table S2).

3.3. OvoA protein sequence analysis and structural modelling

OvoA protein sequences from diatoms *T. pseudonana, P. tricornutum* and *S. marinoi* (both *Sm*OvoA 2388 and 4090 sequences) were aligned with sequences from sea urchins *P. lividus* and *S. purpuratus* (Fig. 2), whose gene and primary protein structure have been defined in detail [13], to assess if the canonical metazoan domain architecture was also conserved in diatoms. The sequence alignment revealed that OvoA from diatoms (2388 sequence for *Sm*OvoA) contains the same bifunctional architecture as metazoan OvoAs: the N-terminal DinB-like domain and the central FGE-sulfatase domain (both responsible for the sulfoxide

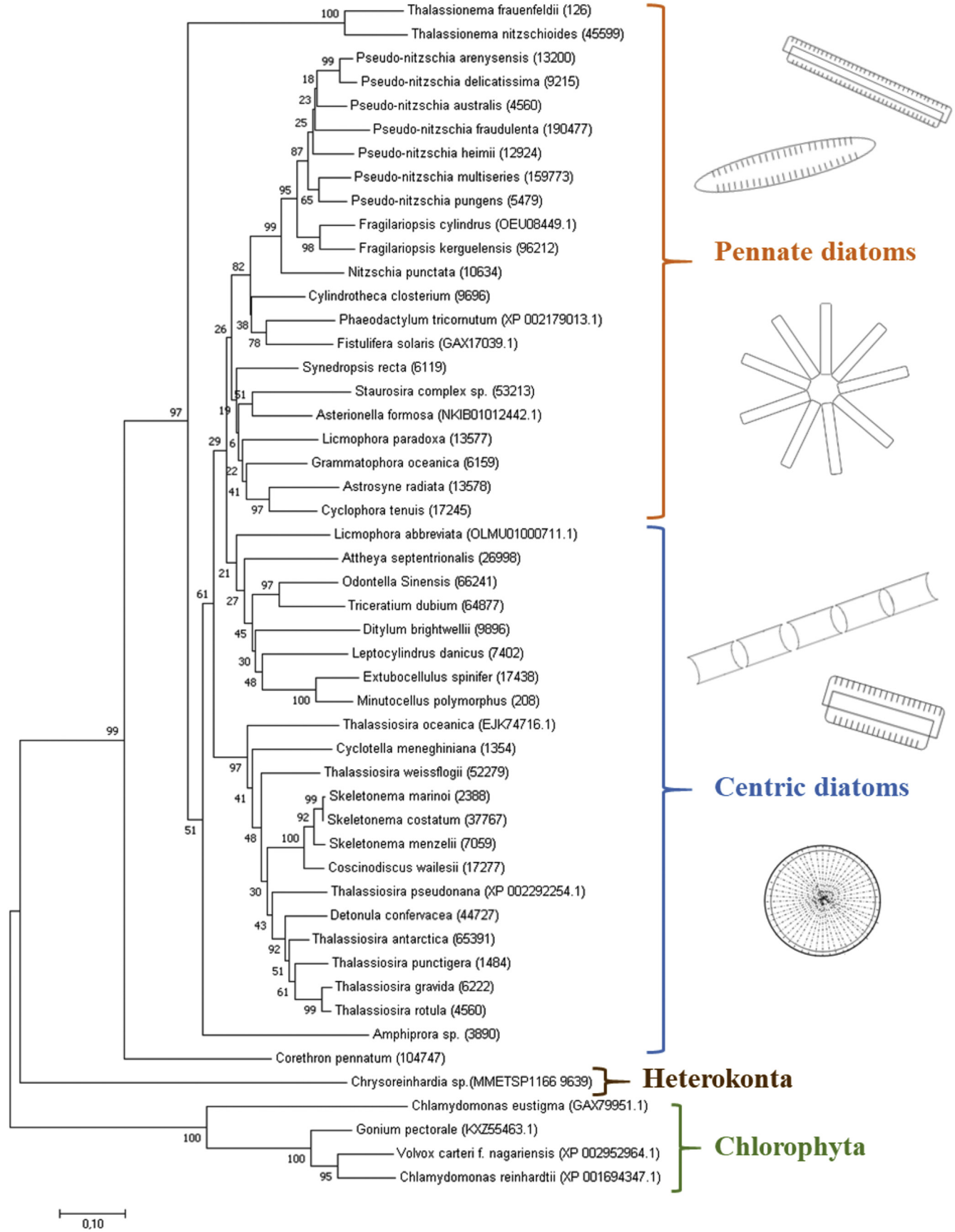

Fig. 1. Neighbor joining tree of OvoA homologs in diatoms. The phylogenetic analysis of OvoA sequences from diatoms was inferred using the Neighbor-Joining method [52]. The optimal tree with the sum of branch length = 6.62215866 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Diatom drawings are not in scale.

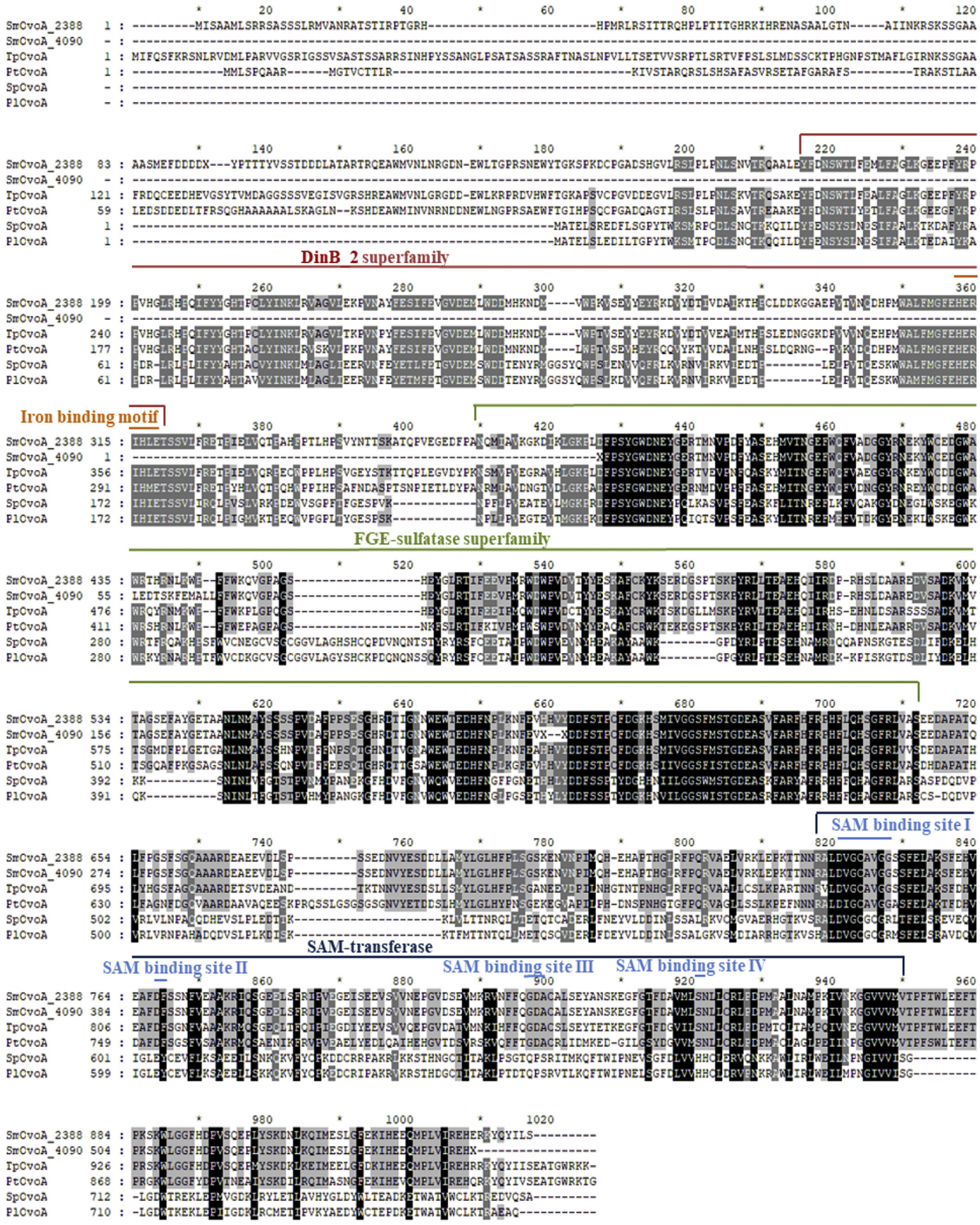

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Fig. 2. Multiple sequence alignment of OvoA proteins from diatoms with metazoan homologs. The OvoA sequences from three species of diatoms (*T. pseudonana*, *P. tricornutum* and *S. marinoi*) and two species of sea urchins (*P. lividus* and *S. purpuratus*) were aligned using ClustalX and Genedoc software. Black, dark-gray and light-gray boxes indicate 100, 80 and 60% residues conservation, respectively. Amino acids that do not share similarity are unshaded. The OvoA canonical domains, DinB superfamily, FGE-sulfatase and SAM-transferase, are indicated as well as the putative binding sites for iron and SAM. Abbreviations: PlOvoA = *Paracentrotus lividus* OvoA; PtOvoA = *Phaeodactylum tricornutum* OvoA; SmOvoA = *Skeletonema marinoi* OvoA; SpOvoA = *Strongylocentrotus purpuratus* OvoA; TpOvoA = *Thalassiosira pseudonana* OvoA.

synthase activity) and the C-terminal SAM-dependent MT (responsible for the methylation on the imidazole ring) (Fig. 2).

The two *Sm*OvoA sequences share 97% identity on 65% of covering (Supplementary Table S1) and, differently from *Sm*OvoA_2388, the 4090 sequence lacks the DinB domain, which contains the putative iron-binding motif (consensus sequence: HX_3HXE). Given the importance of iron for the sulfoxide synthase-catalyzed reaction, it is most likely that ovoA_4090 represents a pseudogene, or has evolved to adapt an ovothiol-unrelated function. Thus, for simplicity, *Sm*OvoA will be referred to the complete transcript 2388. The OvoA sequence identity between *Sm*OvoA and sequences from species that are, to some extent, evolutionarily related to diatoms, such as cyanobacteria and algae, has been evaluated. Interestingly, OvoA from diatoms shares a high degree of conservation with cyanobacterial homologs, including *Moorea producens* (WP_083374139.1) and *Okeania hirsuta* (WP_124145403.1), which share 48% and 45% sequence identity with *Sm*OvoA_2388, respectively, as well as with bacteria like *Candidatus Competibacteraceae bacterium* (HCK80687.1) and *Rhodocyclaceae bacterium* (TXG78338.1), both sharing 49% identity with *Sm*OvoA. A slightly lower degree of conservation of *Sm*OvoA_2388 was found with OvoA from green algae (43% identity with *Chlamydomonas reinhardtii,* XP_001694347.1), red algae (36% identity with *Madagascaria erythrocladiodes,* MMETSP1450_8888), brown algae (41% identity with *Ectocarpus siliculosus*, CBJ49246.1). An OvoA-like sequence was also found in plant parasites called oomycetes (49% identity with *Phytophthora parasitica,* ETK91761.1). This sequence lacks the C-terminal MT domain and is often annotated as EgtB. However, sequences from oomycetes share higher identity with OvoA (> 40%) compared to EgtB (24% identity-with *Chloracidobacterium thermophilum,* 6QKJ). No OvoA was found in higher plants.

The binding sites of OvoA, including the residues recognizing the substrates (cysteine and histidine), have still not been experimentally determined, as well as no crystal structure is available for OvoA. In order to predict the tridimensional conformation of the active site of the enzyme, the *Sm*OvoA tertiary structure has been predicted by homology modelling (Fig. 3). In particular, *Sm*OvoA has been modelled, using the crystal structure of the sulfoxide synthase responsible for ergothioneine biosynthesis, EgtB from *Chloracidobacterium thermophilum* (*Cth*EgtB) as template (Fig. 3B). Since EgtB and OvoA enzymes only share the N-terminal region, including DinB and FGE domains, MT domain of *Sm*OvoA was excluded from the structural alignment with *Cth*EgtB. Stampfli and colleagues [51] have recently identified in the active site of *Cth*EgtB, the key residues being involved in binding to iron (H62, H153 and H157), to the EgtB substrate trimethylhistidine (TMH) (Y385 for binding to the imidazole ring of TMH; Q156, N414, F415 and F416, for interactions with the N-α-trimethylamino moiety) and cysteine (R103, R106, F416 and A420). The modelled active site of *Sm*OvoA resembles the *Cth*EgtB tertiary structure, with the presence of the histidine triad (H212, H311, H315), responsible to coordinate the iron atom, as well the tyrosine (Y594) responsible for the binding to the imidazole ring of histidine (Fig. 3). On the other hand, key differences can be highlighted. In particular, regarding the binding site to histidine, although the two phenylalanines involved in binding to TMH in *Cth*EgtB are conserved in *Sm*OvoA (F627, F629), Q156 and N414 of *Cth*EgtB are substituted by H628 and I314 in *Sm*OvoA, presumably due to the difference in substrate specificity between the two enzymes (Fig. 3B, C). Surprisingly, also the residues involved in cysteine binding in *Cth*EgtB (R103, R106, and A420) are not conserved in *Sm*OvoA (D250, K253, F633) except for the F416 of *Cth*EgtB, which is conserved in *Sm*OvoA (F629) (Fig. 3B, C).

3.4. Thiol compounds analysis in S. marinoi and identification of ovothiol B

The ovoA occurrence in *S. marinoi* suggested that this organism produces ovothiol. To test this hypothesis, we examined the thiol content of *S. marinoi* cells cultured under non stressful light conditions and collected during their exponential phase. Extracted thiols were alkylated with the thio-specific electrophilic dye 4-bromomethyl-7-methoxycoumarin (BMC) and analyzed by reversed phase HPLC. Glutathione was identified as the main thiol with a concentration of 800 ng/mg dry weight, corresponding to an intracellular concentration of 1 ± 0.3 mM. Conversely, no traces (< 5 μM) of known alternative thiols such as ovothiol A or ergothioneine were detected. However, a signal was identified as the BCM-alkylated derivatives of ovothiol B by HR-ESI-MS (m/z_calc. = 404.1275, measured m/z = 404.1258, Δ < 5 ppm) and by co-elution with an authentic sample (Fig. 4). The obtained concentration (40 ng/mg dry material) suggested that the intracellular concentration of ovothiol B in *S. marinoi* was around 50 ± 10 μM. These observations indicated that, under healthy state conditions, ovothiol B was the thiol produced by *S. marinoi* at micromolar concentration compared to the millimolar levels of glutathione.

4. Discussion

Ovothiols are sulfur-containing histidines being receiving an increasing interest due to their beneficial properties for human health [5–10]. They are mainly present in marine environment in a wide range of organisms, from photosynthetic microbes to invertebrates [1]. However, a lack of data on the distribution of its biosynthesis in the microalgal world is substantial. The key enzyme – OvoA – involved in ovothiol biosynthesis is still poorly investigated. So far, only homologs from the bacterium *E. tasmaniensis* and the protist *T. cruzi* have been directly characterized by *in vitro* reconstitution [19,55,56]. Yet, crystal structure is not available for this enzyme, enhancing the difficulty of a reliable identification of OvoA homologs based on genome data.

On the other hand, the highly conserved bifunctional architecture of OvoA with an N-terminal sulfoxide synthase and a C-terminal methyltransferase domain, together with the clear identification of OvoA sequences by comparison with other sulfoxide synthases (EgtB) [57], allowed us to reconstruct OvoA phylogeny in diatoms.

Considering the high degree of conservation among diatom sequences and cyanobacterial ones, it is likely that the *OvoA* gene in diatoms was acquired from the first endosymbiotic event with cyanobacteria. According to this hypothesis, the OvoA is conserved in green and red algae. To the best of our knowledge, OvoA has never been found in higher plants, suggesting a gene loss event in this kingdom, while OvoA was conserved in diatoms, brown algae and oomycetes. The absence of OvoA in plants is very intriguing, suggesting that, like in the animal kingdom, OvoA was likely lost along massive gene loss events during evolution [22].

A further interesting consideration is that ovothiol was recently found in the eye lenses of two species of bony fish [11], although the genes responsible for its biosynthesis were not found yet [13,22]. This may suggest the presence of alternative biosynthetic pathways for ovothiol production or mechanisms of ovothiol uptake from dietary sources. Such an explanation may be relevant for the transfer of this key

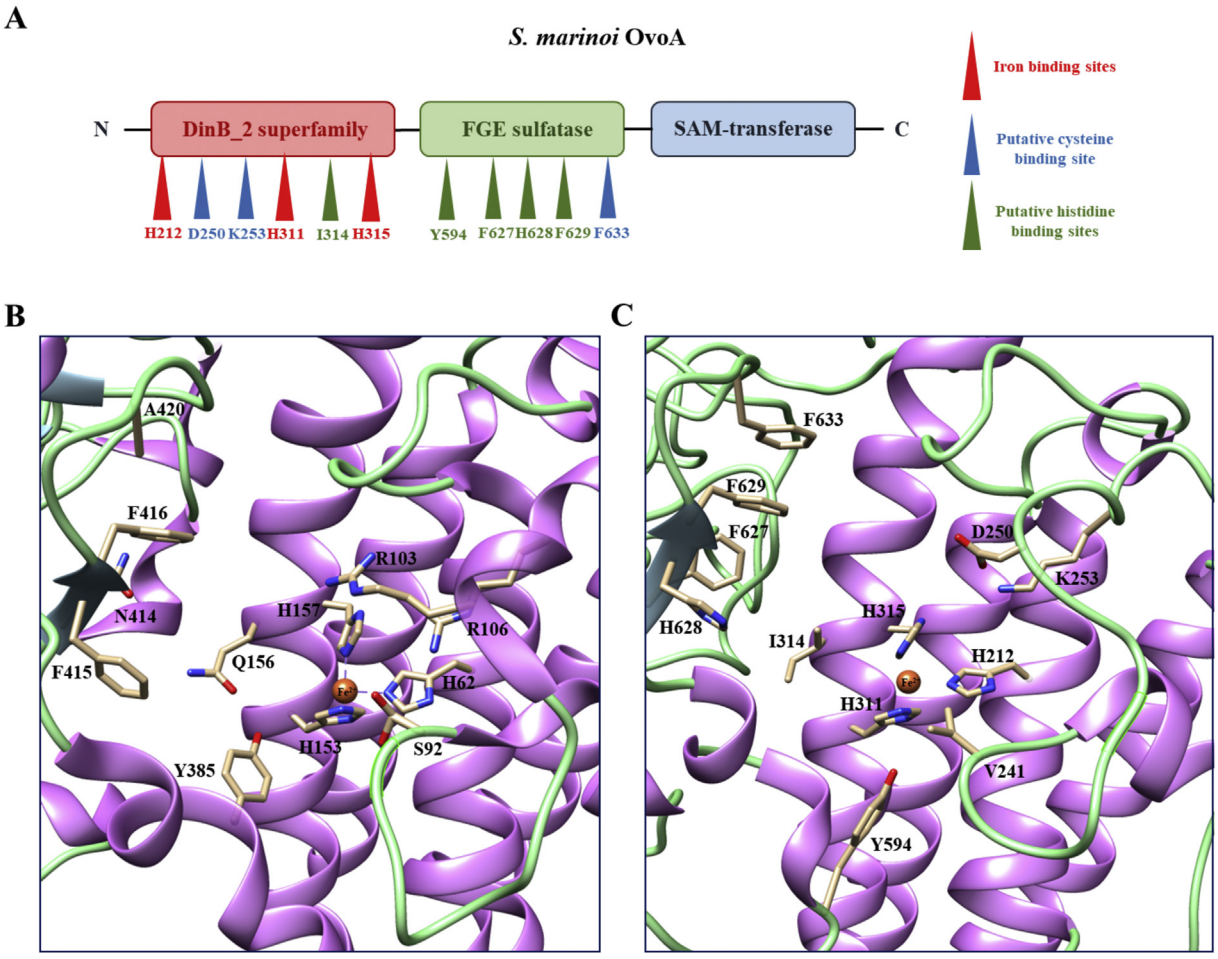

Fig. 3. *Sm*OvoA domain architecture and structural model. A. Scheme of the domain architecture of *Sm*OvoA, including the putative binding sites; **B.** Active site of *Cth*EgtB; **C.** Structural model of *Sm*OvoA.

bioactive compound along the food web at sea from plankton to fish.

The phylogenetic tree of OvoA homologs displays two clades, corresponding to the centric and pennate diatoms. This suggests that, following the acquisition of the gene from the diatom progenitor, OvoA protein diversified according to the phylogeny of the two morphological groups [29].

The absence of ovoA transcript in some diatom species or in some strains of the same species or under particular growth conditions suggests that *ovoA* expression is highly modulated in this group of microalgae, although we have to consider that possible incomplete transcriptome assembling could lead to misleading conclusions. Another possibility could be that *ovoA* gene has been lost in some diatom species, presumably following adaptation to local environmental conditions and speciation processes. However, the still limited diatom

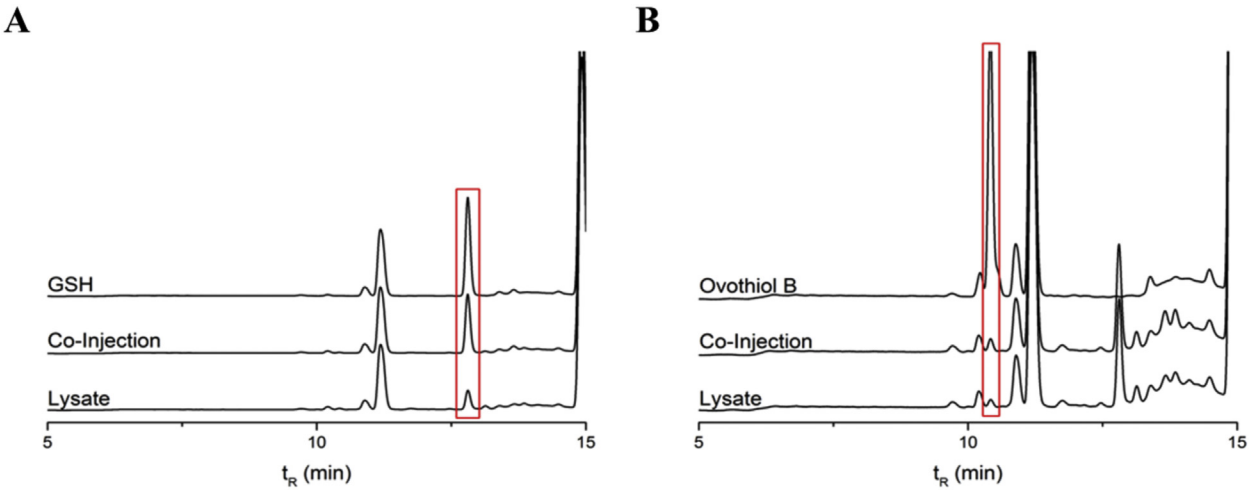

Fig. 4. Thiol compounds analysis in *S. marinoi*. BMC-labeled thiols from *S. marinoi* were analyzed by reversed-phase HPLC. GSH **(A)** and ovothiol B **(B)** were identified in *S. marinoi* lysates by co-injection with authentic standards.

genomes availability does not allow us, for now, to investigate this hypothesis.

We modelled the tertiary structure of OvoA from *S. marinoi* (*Sm*OvoA), a coastal centric diatom considered a very good model for biotechnological applications [35,36], through structural alignment with the ergothioneine biosynthetic enzyme *Cth*EgtB [51]. Our model of the *Sm*OvoA active site revealed a high degree of conservation of the histidine triad, responsible for the coordination of the iron atom, essential for the enzymatic reaction [19], and the key tyrosine, involved in binding to the imidazole ring of the substrate histidine [56]. However, the residues that in *Cth*EgtB are responsible for the binding to the N-α-trimethylamino moiety of TMH and those involved in cysteine binding, were not conserved in *Sm*OvoA. While it was conceivable that residues for histidine binding were not conserved between *Sm*OvoA and *Cth*EgtB, which uses TMH instead of histidine, it was surprisingly to find no conservation also in the cysteine binding residues. This could be ascribed to the fact that the two enzymes are evolutionary distant. Further studies aimed to obtain the crystal structure for OvoA are greatly encouraged for the increasing interest in this class of enzymes.

To fully characterize the ovothiol biosynthesis in diatoms, the thiol fraction purified by *S. marinoi* cells was analyzed for ovothiols content by reversed phase HPLC. Co-elution with authentic standard and HR-ESI-MS analysis revealed a peak corresponding to ovothiol B. This N-methylated form of ovothiol is quite rare in nature, being so far only isolated from the scallop *Chlamys hastata* [58]. The identification of ovothiol B in *S. marinoi* represents the first evidence and characterization of this kind of molecules in diatoms. This finding raises key issues and questions.

First, the concentration of ovothiol B (50 μM) in the diatom is 20 times lower than that of glutathione, differently from what occurs in sea urchins where ovothiol is produced in millimolar concentrations and in higher amounts compared to glutathione [59]. This finding enhances the need for future studies aimed both to understand the biological role in this system, and to find a way to up-regulate its biosynthesis in diatoms for biotechnological purposes. Considering the powerful antioxidant properties of these molecules, we might expect an increase of ovothiol concentration when cells require antioxidant activity enhancement, presumably acting in synergy with other antioxidant molecules, like carotenoids, phenolic compounds, flavonoids, and vitamins, present in microalgae [35,36]. Future studies are required to assess if ovothiol plays a role as antioxidant in this system or it acts as a signaling molecule in fundamental biological processes, by following, for instance, its concentration during exposure of diatoms to environmental condition variations and/or stress. In this context, it has been shown that light intensity and spectral composition strongly induces modulation of the photoprotective and antioxidant networks in diatoms [35,36]. Thus, a light-dependent modulation of the ovothiol biosynthetic pathway could be investigated. Genetic manipulations, aimed to enhance the production of such molecule, could also offer a useful way to increase ovothiol yield in diatoms.

Second, a screening aimed at the chemical characterization of ovothiols produced by other diatoms, both centric and pennate, and more in general in the main microalgal groups, could be carried out, in order to investigate the chemodiversity of ovothiols along a large scale of biodiversity. Then, it might be interesting to investigate the modulation of *OvoA* expression and ovothiol production in target microalgal species (e.g. the most promising from a biotechnological point of view) in response to growth conditions variations, with the aim to enhance its synthesis. This step is needed in the idea of using microalgae (diatoms) as biofactory for bioactive compounds production useful for nutraceutical or pharmaceutical applications.

Third, ovothiol B is the only 5-thiohistidine whose biological activity has never been explored so far, therefore further studies are necessary to assess its bioactivity in human systems. However, both ovothiol A and C have been shown to exert anti-proliferative activity in human cancer cell lines [5,6], hence the methylation at the α-amino group seems to not affect the bioactivity of these molecules.

5. Conclusions

In conclusion, in this work we provided the first identification and characterization of the 5-thiohistidine derivative ovothiol B in a marine centric diatom, i.e. *Skeletonema marinoi*. The exploitation of marine organisms, as sources of bioactive compounds, is becoming increasingly interesting for drug discovery projects. In this context, diatoms could provide a novel eco-sustainable source of ovothiols, necessary for applied research. Future studies should provide new insights into the biological role of these molecules in diatoms and the possibility to increase ovothiol biosynthesis in this model. Yet, the assessment of the bioactivity of ovothiol B in human systems will give insight on the relevance of the methylation at the α-amino group on the beneficial properties of 5-thiohistidines.

Author contributions

AM, IC, CB, AP conceived and designed the experiments. AM, RB performed the experiments. AM, IC, RB, FPS, AP analyzed the data. AM, AP drafted the article. All authors revised and approved the final manuscript.

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Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

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