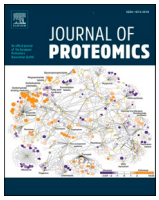
Journal of Proteomics 294 (2024) 105087

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot



Proteomic analysis of the mucus of the photosynthetic sea slug *Elysia crispata*

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ARTICLE INFO

Keywords:
Elysia crispata
Kleptoplasty
Mucus
Proteomics
HPLC-MS/MS
Blue biotechnology

ABSTRACT

Elysia crispata is a tropical sea slug that can retain intracellular functional chloroplasts from its algae prey, a mechanism termed kleptoplasty. This sea slug, like other gastropods, secretes mucus, a viscous secretion with multiple functions, including lubrication, protection, and locomotion. This study presents the first comprehensive analysis of the mucus proteome of the sea slug *E. crispata* using gel electrophoresis and HPLC-MS/MS. We identified 306 proteins in the mucus secretions of this animal, despite the limited entries for *E. crispata* in the Uniprot database. The functional annotation of the mucus proteome using Gene Ontology identified proteins involved in different functions such as hydrolase activity (molecular function), carbohydrate-derived metabolic processes (biological processes) and cytoskeletal organization (cell component). Moreover, a high proportion of proteins with enzymatic activity in the mucus of *E. crispata* suggests potential biotechnological applications including antimicrobial and antitumor activities. Putative antimicrobial properties are reinforced by the high abundance of hydrolases. This study also identified proteins common in mucus samples from various species, supporting a common mechanism of mucus in protecting cells and tissues while facilitating animal movement.
Significance: Marine species are increasingly drawing the interest of researchers for their role in discovering new bioactive compounds. The study "Proteomic Analysis of the Mucus of the Photosynthetic Sea Slug *Elysia crispata*" is a pioneering effort that uncovers the complex protein content in this fascinating sea slug's mucus.
This detailed proteomic study has revealed proteins with potential use in biotechnology, particularly for antimicrobial and antitumor purposes. This research is a first step in exploring the possibilities within the mucus of *Elysia crispata*, suggesting the potential for new drug discoveries. These findings could be crucial in developing treatments for severe diseases, especially those caused by multidrug-resistant bacteria, and may lead to significant advances in medical research.

1. Introduction

Sacoglossa is a superorder of sea slugs that possess a few species with a unique ability to retain functional chloroplasts – kleptoplasts – from ingested algae [1]. This process, known as kleptoplasty, enables the sea slug to utilize photosynthesis for its nutrition [2]. The capacity to steal chloroplasts is common in single-celled eukaryotes, such as foraminiferans, dinoflagellates and ciliates, but a rarer evolutionary trait in Metazoa. In animals, kleptoplasty has been identified exclusively in flatworms and sea slugs [3,4]. *Elysia crispata* (Mörch, 1863) is a tropical kleptoplastidic sea slug commonly referred to as the "lettuce sea slug" due to its green colour and undulating parapodia [5]. This sea slug is known to feed on a wide range of ulvophycean algae species, mainly from the genera *Bryopsis*, *Halimeda* and *Penicillus* [6,7]. Carbon supply from kleptoplast photosynthesis was shown to be quantitatively important for mucus synthesis in *E. crispata* [8,9].

Sea slugs mucus serves various functions, including protection or defence, locomotion and nutrition [10]. Gastropod mucus has a complex biochemical composition that varies across species [11]. Polysaccharides, proteins, lipids and minerals are some of the key

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https://doi.org/10.1016/j.jprot.2024.105087
Received 5 December 2023; Received in revised form 11 January 2024; Accepted 15 January 2024
Available online 17 January 2024

components found in gastropod mucus [12]. Mucin proteins, a significant group of proteins present in gastropod mucus, are large highly glycosylated proteins that contribute to the viscosity and protective properties of the mucus [13]. Mucins are among the variety of proteins produced in mucus by marine animals such as sponges, bryozoans, and gastropods that also help prevent the animal from drying out, reduce friction during movement and protect against potential predators [14,15]. Other proteins found in gastropod mucus include enzymes involved in digestion, anti-predator proteins, and proteins involved in wound healing [11]. Some marine animals also produce enzymes that degrade the mucus of other animals, facilitating the absorption of resulting nutrients [16,17]. Antimicrobial peptides, which are small, cationic proteins, are also present in the mucus. These peptides exhibit toxicity to bacteria, viruses, and other microorganisms thereby preventing infections and protecting the animal from diseases [18–20]. Marine animal mucus can also contain a variety of other proteins, including hormones, growth factors, and structural proteins [21].

Proteomic studies of marine animal mucus have provided important insights regarding the roles played by these proteins and their contribution to the survival of the organism in its habitat. These studies have also revealed the diversity of proteins present in the mucus of various species, highlighting the importance of sustained research in this field. The focus of this study is to characterize the mucus proteome of the sea slug *Elysia crispata*. By comparing the mucus proteome of *E. crispata* with those of other species, this study aims to highlight the unique functions and adaptations of the proteins present in the mucus of this particular species.

2. Materials and methods

2.1. Animal maintenance

Elysia crispata was successfully bred in the laboratory from an initial pool of 20 adults, collected in Florida, USA, which were purchased from Tropical Marine Centre Iberia (Lisbon, Portugal). Animals were kept in a 150 L recirculated life-support system (LSS) with artificial seawater (ASW) with a salinity of 35 ppt and a temperature of 25 °C. ASW was prepared by mixing salt (Red Sea Europe, Verneuil d'Avre et d'Iton, France) with reverse osmose water (TMC, V2 Pure Advanced Reverse Osmosis System).

The LSS was equipped with T5 fluorescent lamps with a photon scalar irradiance of 60 μmol photons m^{-2} s^{-1} measured at the water surface and set for a photoperiod of 12 h light:12 h dark. The sea slugs were fed with the alga *Bryopsis plumosa* (strain KU-0990, acquired in 2018 from the Kobe University Macro-Algal Culture Collection, Japan). The alga was grown at 20 °C, in 2 L flasks with ASW and f/2 medium, without silica, with constant aeration. The alga was exposed to an irradiance of 60–80 μmol photons m^{-2} s^{-1} provided by LED lamps (Valoya 35 W, spectrum NS12) and a photoperiod of 12 h light:12 h dark.

2.2. Mucus collection

First-generation laboratory-reared *E. crispata* adults were randomly sampled from the LSS for mucus collection. Animals were passed through clean ASW to remove any debris attached to the sea slugs. Each animal was then placed in a sieve and excess water was removed with tissue paper. The slugs were then placed in individual 8 cm watch glasses, wetted with 200 μL of ASW and gently swirled for 5 min to induce mucus production. The secreted mucus was then collected with a micropipette into previously cooled 15 mL falcon tubes. Each mucus sample represented a pool of 7 individual sea slugs and a total of 3 samples were collected for analysis. Samples were then frozen at −80 °C for further processing.

2.3. Sample preparation for proteomics

The protocol used for protein extraction was adapted from Kang et al. [22]. Samples were thawed and homogenized for 5 min in an ultrasonic bath (VWR, Radnor, USA) at room temperature. 300 μL of extraction buffer (1.5 M Tris-HCL, pH 8.5; 5 M Urea; 2% SDS; 2 mM DTT) were added to 900 μL of sample. To ensure homogenization, each sample was vortexed for 1 min followed by 15 min sonication (VWR, Radnor, USA). Samples were then incubated for 1 h at 60 °C and 400 rpm. After cooling to room temperature, 50 μL of a 5.5 mM iodoacetamide (IAA) solution was added to each sample and left in the dark for 20 min at room temperature. To stop the reaction, 50 μL of β-mercaptoethanol were added to each sample and centrifuged at 4 °C and 24,500 x g for 15 min. The supernatant was collected in a new tube to which acetone at −20 °C was added in a ratio of 1:4 and the solution was allowed to precipitate overnight at −20 °C. The samples were then centrifuged at 4 °C and 24,500 ×g for 15 min and the precipitate was resuspended in the extraction buffer. The protein concentration was determined with the RC/DC protein assay kit (BioRad, Herculer, CA, USA) according to the manufacturer's protocol. After quantification, samples were prepared for gel electrophoresis.

2.4. Gel electrophoresis and peptide sequencing by mass spectrometry

Sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis and in-gel digestion were performed as previously described [23]. For gel electrophoresis, a loading gel buffer (0.5 M Tris, pH 6.8; 10% SDS; 100% glycerol; bromophenol blue and β-mercaptoethanol) was added to the protein extracts, incubated for 5 min, at 95 °C and 300 rpm. Samples were then loaded into 10% SDS gels. The gel was run for 1 h, at 180 V, using a Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad, California, USA) and then stained with Coomassie Blue (Bio-Rad, California, USA). The gel was divided into smaller portions and destained through multiple washes using the following solutions: 25 mM Ammonium Bicarbonate (AMBIC), 50% Acetonitrile (ACN)/25 mM AMBIC, and 100% ACN. After the washing step, the gel pieces were incubated at 56 °C for 45 min with a 10 mM DTT in 25 mM AMBIC solution.

Subsequently, the solution was replaced with a 55 mM IAA in 25 mM AMBIC solution and incubated at room temperature in the dark for 30 min. Next, the gel pieces were washed again using the same solution as the previous washing step. A solution of trypsin in 50 mM AMBIC was added to the gel pieces and incubated on ice for 45 min. The excess trypsin was removed, and the gel pieces were completely covered with 50 mM AMBIC and left overnight. The supernatant was collected into new vials and subjected to two washes: the first with a 5% formic acid (FA) solution for 20 min, followed by a second wash with a 5% FA in 50% ACN for 20 min. The samples were then partially dried using a speed vacuum (Savant SPD121P SpeedVac Concentrator, Thermo Fisher Scientific, Bremen, Germany). Finally, a 1% FA solution was added to each sample. The peptides were then analysed by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). Samples were loaded onto an EASY-Spray HPLC column (75 μm × 150 mm, 2 μm, 100 A, Thermo Fisher Scientific, Bremen, Germany), at 35 °C, and separated using a Nanoflow HPLC (Ultimate 3000 Dionex, Thermo Fisher Scientific, Bremen, Germany), coupled to a Q-Exactive hybrid quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The buffer system consisted of two mobile phases (buffer A- 0.1% FA in HPLC grade water; buffer B- 80% HPLC grade ACN in 20% HPLC grade water and 0.1% FA) and the flow rate was 300 nL/min. Elution of peptide was performed using a 100 min gradient (0–3 min of 96% solvent, 3–70 min of 4–25% solvent B, 70–90 min of 25–40% solvent B, 90–92 min of 90% solvent B, 92–100 min of solvent B and 101–120 min of 96% solvent A) with a flow rate of 300 nL/min. Data acquisition was performed using the Xcalibur v3.3 data system (Thermo Fisher Scientific, Bremen, Germany).

2.5. Protein quantification and identification

Proteins were identified using Proteome Discover software (v2.2.0.388, Thermo Fisher Scientific, Bremen, Germany). Data were obtained by searching against the *Elysia* protein database (accessed 21 April 2022, taxon identifier = 71,493, Swiss Prot), with Sequest HT and MS Amanda 2.0 search engines, with percolator validation. For the protein identification, the following parameters were used: carbamidomethylation of cysteine, as a static modification, acetylation of N-terminal proteins (N-Terminus) and oxidation of methionine as dynamic modifications. The mass tolerance for precursor and fragment ions was 10 ppm (MS) and 0.02 Da (MS/MS). For trypsin digestion, up to two missed cleavages were considered, while the threshold of the global false discovery rate (FDR) for peptides and proteins was 0.01. For data analysis, only proteins with a minimum number of two peptides were considered. Proteins were quantified using a label-free method, using the area of the total ion current of the precursor ion.

2.6. Gene ontology analysis and enzymatic activity

Functional annotation was conducted via OmicsBox software (v2.1.14, Biobam, Valencia, Spain), using the Functional Analysis Module which is accessible at https://www.biobam.com/omicsbox/#functional. The list of annotated protein sequences was imported into OmicsBox to perform Gene Ontology (GO) annotation of the molecular function of proteins, cellular components and biological processes and mapping. The protein sequences with annotations were aligned to the *Elysia* sequence obtained from NCBI using a local BLASTP with default parameters and an expected value of 1e-25. The results from this search were used for downstream analyses, including mapping, annotation, gene ontology, and pathway analysis, using default values. Subsequently, the GO analysis was classified into three categories, namely cellular components, molecular function, and biological process.

2.7. Protein expression

To evaluate protein expression levels and presence in mucus samples, a search was conducted in Proteome Discover using the transcriptome of *Elysia chlorotica* [24] using the same parameters described for protein identification. The proteins reported in the transcriptome were matched to the proteins found in mucus samples, and the corresponding FASTA sequences of the matched proteins were searched in the *Elysia* Uniprot database (accessed 21 April 2022, taxon identifier = 71,493, Swiss Prot). Subsequently, a search for the Accession Numbers of the identified proteins in the transcriptome database (mRNA database) was carried out. After searching all Accession Numbers, both databases were compared to determine the proteins present in the mucus.

2.8. Statistical analysis

Multivariate analysis was performed using R V4.2.2 (R Core Team, 2021) in RStudio 2022.12 (RStudio Team, 2020). The data was normalized by log-transformation of the values. Missing values were disregarded for the analysis, resulting in a loss of 15% of overall proteins. To evaluate the protein abundance among samples and technical replicates, a PerMANOVA was performed with the R package vegan [25] and the *p*-values were based on 999 permutations. Heatmaps were generated using the R package ComplexHeatmap [26] and "Euclidian" as the clustering distance and "complete linkage" as the clustering method. Additionally, a Friedman test was conducted with the R datarim package [27] to assess differences in protein abundance within samples.

2.9. Evolutionary analysis by maximum likelihood method

Evolutionary analyses were performed using the MEGA11 software [28] and the resulting tree was uploaded to iTOL v5 [29] for further analysis and visualization. The evolutionary history was determined using the Maximum Likelihood method and a JTT matrix-based model [30]. The initial tree(s) for the heuristic search were generated automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, followed by the selection of the topology with the highest log-likelihood value. A total of 306 amino acid sequences were analysed and the final dataset contained 5606 positions.

2.10. H&E staining

To assess cellular shedding and other forms of contamination, mucus samples stained with the H&E stain kit (Merck, Darmstadt, Germany) were observed. Samples were visualized with the inverted microscope Leica DMi1(Leica Microsystems GmbH, Wetzlar, Germany) and analysed with Leica Application Suite X 5.0.3 (Leica Microsystems GmbH, Wetzlar, Germany).

3. Results and discussion

In this work, we report for the first time the mucus proteome of *Elysia crispata*. To establish a comprehensive protein expression database, we aimed to collect data under minimal stress conditions at the basal level of mucus production. To achieve this, we collected three samples, each comprising a pool of mucus secreted by seven animals grown under identical conditions, as described in the Materials and Methods section. Subsequently, these samples were processed for protein extraction and separation by gel electrophoresis.

3.1. Mucus protein separation and identification by mass spectrometry

Following gel electrophoresis, 11 major protein bands with molecular weights estimated between 75 and 11 kDa were identified (Supplementary Fig. S1). No observable variations were detected between the replicates, indicating the reproducibility of the mucus extraction technique. The most intense protein bands were observed at 75 kDa, 63 kDa, 35 kDa, between 20 and 25 kDa, between 17 and 11 kDa, and <11 kDa. Gels were then processed for HPLC-MS/MS analysis as described in Materials and Methods, yielding a total of 9 samples consisting of three biological replicates (A, B and C) and three technical replicates (1, 2 and 3).

Li and Graham [31] reported 14 distinct proteins bands in the mucus of land slug *Lehmannia valentiana*, with the most intense bands ranging from 300 KDa to 30 KDa (300 kDa, 200 kDa, 130 kDa, 85 kDa, 65 kDa, 52 kDa, 40 kDa and 30 kDa). SDS gel electrophoresis of mucus proteins from the land gastropods *Cernuella virgata, Cochlicella acuta, Cochlicella barbara* and *Theba pisana* exhibited fewer bands, with molecular weights ranging from 300 kDa to 55 kDa, with the most intense bands observed between 100 kDa to 76 kDa [32]. When compared to the bands from *E. crispata*, a noticeable difference in the molecular weight ranges was detected, with mucus samples of *E. crispata* exhibiting a lower molecular weight range. However, when compared to the marsh periwinkle sea snail *Littorina irrorate* [33] the mucus secretions had a protein molecular weight range similar to *E. crispata* samples, between 65 kDa and 36 kDa.

Following SDS-gel electrophoresis, the samples were analysed for protein identification analysis, as described in Materials and Methods. In this study of *E. crispata*, we identified proteins using homology, as no genome for this species has been described. We obtained all available data from the NCBI database for the genus *Elysia* and analysed the samples using HPLC-MS/MS. Of the 589 proteins identified, we only considered those with two matched peptides (see Materials and Methods section), resulting in a total of 306 identified proteins (see Supplementary Table S1 for details). We considered it possible that skin cells and faecal particles exist in the mucus samples, which could result in an increased number of protein matches that were not part of the mucus. To confirm whether the cells present in the mucus samples were

noteworthy, we conducted an H&E staining test. Based on the smears obtained (see Supplementary Fig. S2), we determined that the cell count was not substantial enough to cause a significant bias in the data. In the application of our peptide identification strategy against the transcriptome of *Elysia chlorotica* [24], our analysis led to the identification of 322 proteins (identified by two or more peptides) (Supplementary Table S2).

In this study, we employed a label-free quantification approach for protein analysis, focusing on the area of the total ion current of the precursor ions. While this method is widely recognized for its efficacy in quantifying proteomes in model organisms, its application in non-model organisms warrants careful consideration. Recent literature has highlighted potential biases inherent in label-free quantification methods, particularly in the context of non-model organisms [34]. These biases primarily arise due to the differential ionization efficiencies of peptides and the complexity of the sample matrix, which can lead to an underrepresentation of certain proteins. To address these challenges and enhance the reliability of our findings, we included in the analysis the identification of peptides against the transcriptome database, allowing for a more comprehensive and accurate representation of the proteome.

While there are some studies on gastropod proteomes, few have focused specifically on mucus. Tachapuripunya et al. [35] analysed mucus samples from seven gastropod species including *Achatina fulica, Pomacea canaliculata, Cryptozona siamensis, Semperula siamensis, Hemiplecta distincta, Cyclophorus fluguratus* and *Semperula siamensis*. Their study identified 1634 proteins across the seven species. Another study analysed the mucus secretions of the land snail *Cornu aspersum* to evaluate the protein content in the different mucus secretions [36]. The authors also sequenced the transcriptome of the land snail and used proteomics sequencing to identify 71 proteins in purified mucus samples. This number is lower than what we observed in the mucus secretions of *E. crispata.* However, other studies have reported higher numbers of proteins in mucus, such as Espinosa et al. [37] who identified 902 proteins in the mucus of the oyster *Crossotrea virginica* and Kang et al. [22] who identified 171 proteins in the pedal mucus of *Patella vulgata* L.

The variability in results is not only dependent on the number of proteins present in the mucus but also on the number of annotations available in the Uniprot database, many of which are incomplete. *Crossotrea virginica*, for example, has a greater number of entries (50,485), which increases the likelihood of a match. Conversely, *Cornu aspersum* only has 305 entries, while *Elysia crispata* has only 31 entries in Uniprot. To obtain a comprehensive identification of the *E. crispata* mucus, we had to broaden the database, resulting in 24,602 entries for the *Elysia* genus. For this reason, at this stage, we cannot be certain that all proteins present in mucus samples of *E. crispata* were identified.

Mucus sampling in *E. crispata* was the most challenging aspect of the study, due to its relatively small amount and viscosity. To determine the reproducibility of the mucus sampling technique, we conducted a PerMANOVA analysis to evaluate the overall centroids and dispersion of the samples, based on protein abundance. There were no significant differences in protein abundance across the samples ($p = 0.25$). The heatmap in Fig. 1 displays the relative abundance of the identified proteins across samples, showing that they are similar, with no significant differences observed between samples. To determine if there were any proteins present that had varying abundance across the different samples, we conducted a Friedman's test for each protein. The test showed no statistical differences in protein abundance between samples ($p > 0.05$). Therefore, we can conclude that the mucus sampling technique is replicable, with no statistically significant differences observed between samples taken at different time points and analysed from different gels.

3.2. Gene ontology and evolutionary analysis by maximum likelihood method

The proteins that were identified were functionally annotated by Gene Ontology (GO) using the Omicsbox software. Only those proteins that were with a GO term were considered for further GO analysis, as the Omicsbox software only accepts annotated sequences for this purpose. The GO analysis retrieves GO terms for gene expression, using the molecular function of the proteins, the biological process they affect, and the cellular location of the molecules. The Omicsbox software provides the ability to group the molecular function of the proteins according to enzymatic code, which is extensive to Enzyme annotation. This tool was used to group the enzymatic activity of mucus proteins according to the main enzyme class (distribution of the main enzyme classes) and a second-level class (subgrouping of the main classes, with more detail on the activity). The results show that the mucus proteome of *E. crispata* is responsible for significant molecular functions such as enzymatic

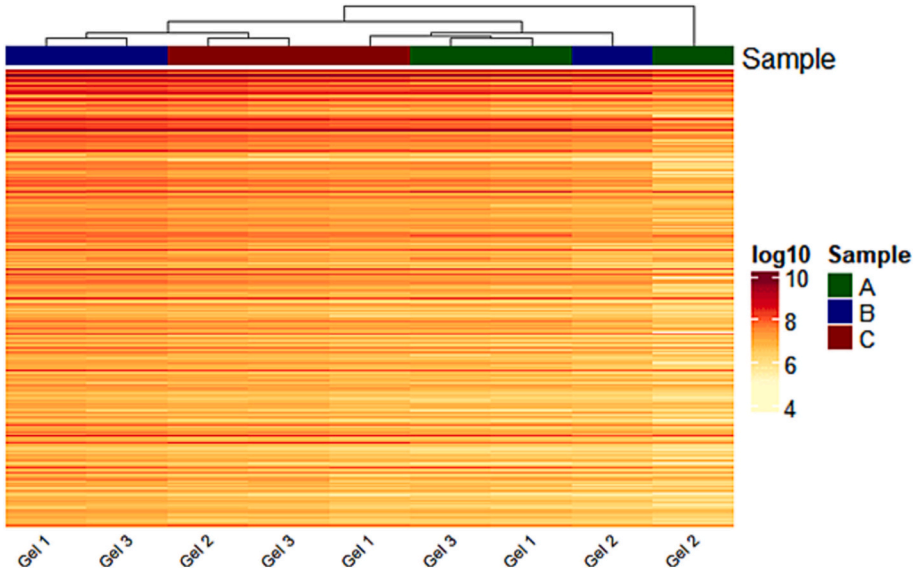

Fig. 1. Heat map of the relative abundance of the proteins identified in samples (A, B, C) and their analytical replicates (Gel 1, Gel 2, Gel3) with sample clustering shown at the top. PerMANOVA $F = 1.2597$, $p = 0.25$. No significant differences were identified between samples. The figure was created using R V4.2.2 (R Core Team, 2021) in RStudio 2022.12 (RStudio Team, 2020), with the R package ComplexHeatmap [26].

activity (Fig. 2 and Supplementary Table S3) as well as binding and transport activities. The high number of proteins associated with the enzymatic activity is consistent with studies that have been conducted on Gastropoda for the search for bioactive compounds with potential applications in health [38–47].

The identified proteins exhibited various functions related to biological processes, such as carbohydrate-derived metabolic processes, cytoskeletal organization, and nucleobase-containing small molecule metabolic processes. These proteins are mainly located in the cytoskeleton, indicating that they are cell-associated. This finding is consistent with previous studies on mucus from other sources, such as Casado et al. [48] who identified that around 52% of all the proteins found in human nasal mucus secretions were located in cellular components.

To provide a better understanding of the identified proteins and their predicted names, we have compiled the results in a table (see Supplementary Table S2) that classifies the main protein families and the number of proteins in *E. crispata* mucus. Our analysis revealed that the mucus of *E. crispata* was composed of 15 types of actins, 12 types of RAS-related proteins, 11 types of tubulin, 11 types of 40s ribosomal proteins, and to a lesser extent, 6 types of proteosome subunit alpha, 5 types of ATP synthase proteins, 5 types of myosin, 5 types of ATPase, 5 types of histones, 4 types of coesterase domain-containing protein, 4 types of WD_REPEATS_REGION domain-containing protein, 4 types of annexin, and 4 types of 60S ribosomal proteins.

Actins are a family of globular proteins responsible for the formation of microfilaments in the cytoskeleton [49] and thin filaments in muscle fibrils [50]. RAS proteins are signal-transducing proteins, that play a major role in cancer in humans, with several studies attempting to understand the mechanism behind RAS protein regulation and cancer [51]. Tubulins are a superfamily of globular proteins that polymerize into microtubules, which are important backbones for the formation of the cytoskeleton in animal cells. Due to the essential role that these proteins play in cell assembly, they have become the subject of research on important diseases such as cancer and Alzheimer's [52].

After comparing the results of this GO analysis with those previously conducted on gastropods and marine animals, we observed similarities in major biological processes, molecular functions, and cellular components, as reported in earlier studies [35]. However, we also identified a greater proportion of proteins with enzymatic activity in the mucus of *E. crispata*. Therefore, we have explored the potential of *E. crispata* mucus as a source of proteins with biotechnological applications, such as antimicrobial and antitumor activities, which could facilitate the development of new applications. Table 1 displays some of the proteins with interesting biological activities that we identified in the *E. crispata*

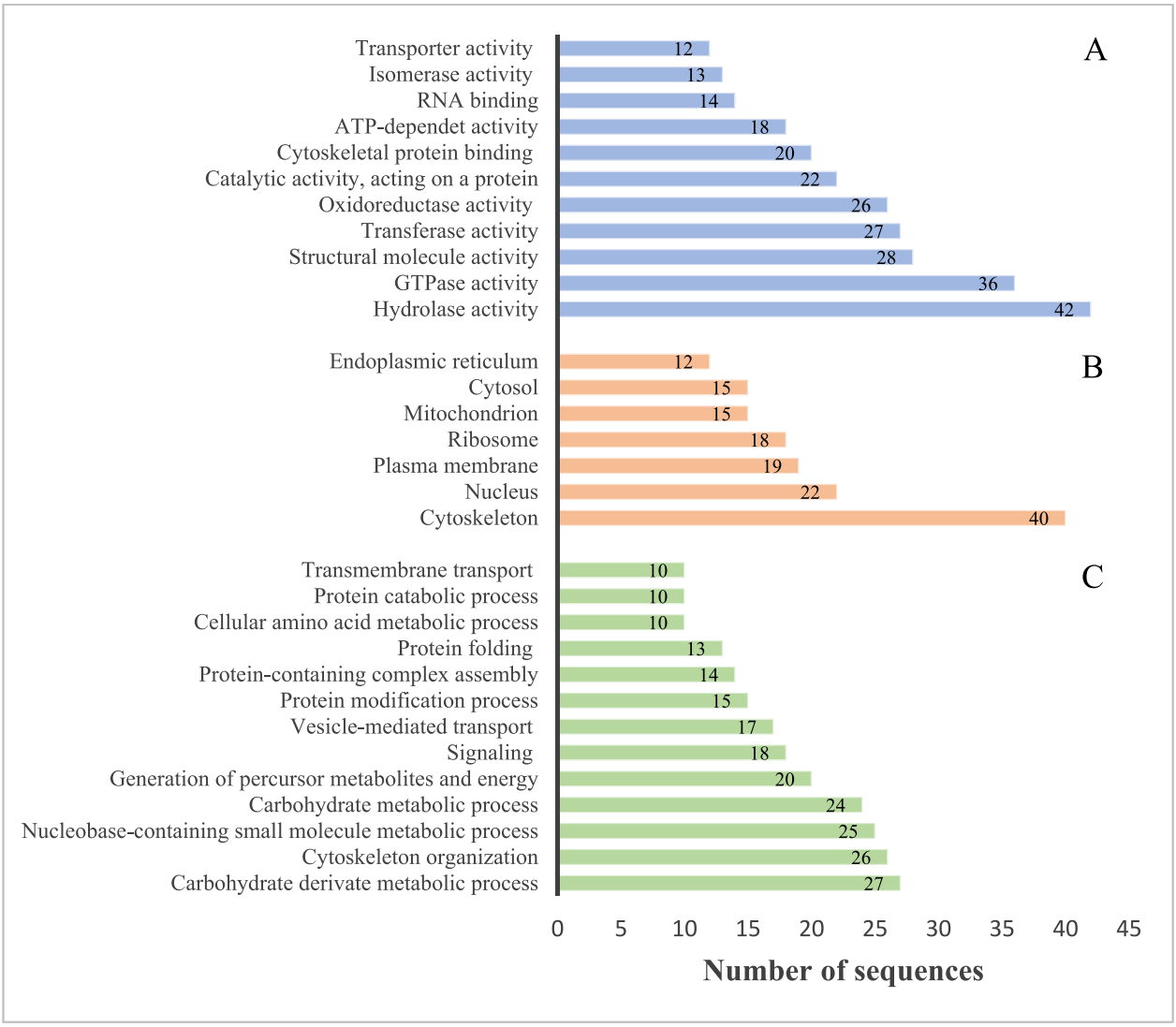

Fig. 2. Gene function classification (Gene Ontology; GO) of proteins identified in the mucus of *Elysia crispata*. The chart represents the number of sequences identified by A) biological process; B) cellular component; and C) molecular function. The analysis was performed on level 3 GO terms assigned by OmicsBox. Only GO terms with at least 10 protein sequences are shown in this figure (for more information, please see Supplementary Table S3).

Table 1
Proteins identified in the mucus of *Elysia crispata* that have potential biotechnological applications accordingly to existing literature. The table provides information on the accession number of each protein in the Uniprot database, the name of the protein, the referenced biotechnology application and relevant bibliographic references.

Accession number	Protein name	Applications	References
A0A433U284	Major vault proteins	Resistance of Human diploid fibroblasts to apoptosis Possible drug delivery system Apoptotic signalling	[53–57]
A0A3S1BM67	Heat shock protein 70	Apoptotic signalling Neuroprotection	[58,59]
A0A433TQC7	Lysozyme	Antimicrobial Apoptotic signalling Anti-inflammatory	[60–62]
A0A3S1BBK1	Dyp-type peroxidase	Hydrolysis Antioxidant Antimicrobial	[63,64]
A0A433TE92	Peroxiredoxin	Antioxidant Potential antitumoral	[65–68]
A0A3S1C043	Thioredoxin domain-containing protein	Antioxidant Possible antiviral Immune regulation	[69–71]
A0A3S0ZCS2	Glutathione transferase	Immune regulation Antioxidant	[72–74]

mucus.

To infer the missing molecular function of proteins, we conducted an evolutionary analysis, by creating a phylogenetic tree of the mucus protein's biological process (Fig. 3) using a Maximum Likelihood method [28], as detailed in the Materials and Methods section. We aimed to retrieve information on the possible biological functions of proteins with missing GO annotations, based on their evolutionary biological relationships with proteins that have known functions. Through this approach, we were able to assign possible functions to specific protein groups. For example, group K was found to be mainly responsible for the protein folding process, group A for the catabolic process of proteins, group J for cytoskeleton organization, group E for signalling and transport, and group B for the cellular amino acid metabolic process. For a detailed view of the proteins in each group and subgroup, please refer to Supplementary Table S3. By using this approach we can analyze proteins without a GO annotation within these groups, and infer its possible function. We then applied this approach to the 20 most abundant proteins and inferred possible molecular functions for proteins without a GO annotation in the Uniprot database.

3.3. Top 20 Most abundant proteins

After analysing the entire dataset of identified proteins, we examined the 20 most abundant proteins, listed in Table 2, to gain insights into the mucus biological activity. Notably, actin (accession number A0A3S1HT72) was identified as the most abundant protein in the mucus of *E. crispata* which is consistent with its well-established abundance in eukaryotic cells [75].

To better understand the functional properties of these 20 most abundant proteins, we analysed their GO annotations. We found that these proteins were primarily predicted to be located in the nucleus and the cytoskeleton, specifically in microtubules. Concerning their molecular function, binding activities such as RNA, DNA, GTP and ATP binding were the predominant functions identified, while for biological processes, the main function was found to be related to microtubule-based processes, primarily involving actin proteins. Notably, we observed a protein, A0A433TPX9, which is described as exhibiting cholinesterase activity [76].

For the three proteins, A0A3S1BB22, A0A3S1BU17, and A0A3S0ZBN5, which did not have a GO annotation or a description, we searched in the maximum likelihood tree to infer their possible molecular function. This approach enabled us to identify protein A0A3S1BB22 in group A, which may indicate its involvement in protein catabolic processes. Similarly, protein A0A3S1BU17 was in group B, suggesting a possible molecular function in the cellular amino acid metabolic process. We were unable to provide a possible function for protein A0A3S0ZBN5 as it was located in group D, which currently has no assigned molecular function.

The protein lysozyme (accession number A0A433TQC7) was found to be the fifth most abundant protein in the mucus of *E. crispata*. This protein has attracted the attention of researchers due to its potential applications in the field of medicine [61]. Several studies have shown that lysozyme exhibits interesting potential as a treatment for infectious diseases, due to its antimicrobial activity and wound-healing properties [60,61]. Lysozyme can be obtained from various sources, including plants, marine invertebrates, insects, milk, and egg white. Therefore, the discovery of this protein in *E. crispata* mucus alone is not a significant finding. However, potential studies on the activity of lysozyme from *E. crispata* mucus may lead to the discovery of specific applications, such as increased antimicrobial activity or activity against particular bacteria.

Little is known about the functions and potential applications of the remaining 20 most abundant proteins, except for actin. Nonetheless, some proteins warrant further studies. For example, MACPF domain-containing protein, which is a protein superfamily known to be involved in bacterial pathogenesis and eukaryotic cell immunity [77]. Zhang et al. [78] found that this protein family can aid in the antibacterial response against pathogens. Limited data are available in the literature regarding the Inhibitor_I29 domain-containing protein. However, since it is an inhibitor protein, it may exhibit interesting inhibitory activity against pathogens. Although little information is available regarding Glyco_hydro_79C domain-containing protein, the GO analysis identified its molecular function as hydrolase activity, suggesting its potential as a molecule of interest for further study.

3.4. Enzymatic activity

We employed the OmicsBox software [79] to determine the potential enzymatic activity in the mucus of *E. crispata*, as outlined in the Materials and Methods section. OmicsBox not only facilitates the identification of the main enzymatic activity but also enables further analysis of the specific activity subgroups within the main enzymatic groups. Our analysis revealed the presence of the seven major enzyme types in the mucus of *E. crispata*: hydrolases (47%), oxidoreductases (16%), transferases (14%), isomerases (7%), translocases (7%), ligases (6%) and lyases (2%) (Fig. 4).

Notably, mucus hydrolases from *E. crispata* accounted for 47% of all proteins with putative enzymatic activity. Hydrolases are a class of proteins that catalyse biochemical hydrolysis reactions, being involved in a wide range of metabolic processes [80]. Among the hydrolases, those acting on acid anhydrides (31.3%) comprise most of the mucus from *E. crispata*, followed by peptidases (4.8%) (Table 3). Peptidases are a well-studied enzyme family, exhibiting diverse applications in biotechnology [81]. Hydrolases are attracting increasing attention for their antimicrobial properties, particularly lysozymes (glycosylases, 4.2%) [37,82–84]. Given that these hydrolase enzymes account for almost half of the proteins found with enzymatic activity, this suggests the need to assess the antimicrobial activity of *Elysia crispata* mucus.

Furthermore, we found that transferases (14%) acting on phosphorus-containing groups (6%) were the second most abundant sub-group of enzymes in *E. crispata* mucus. A transferase is an enzyme that facilitates the transfer of distinct functional groups, such as methyl or glycosyl groups, from a donor molecule to an acceptor molecule. This class of enzymes is essential to numerous biochemical pathways in biology, playing a critical role in many of life's fundamental processes

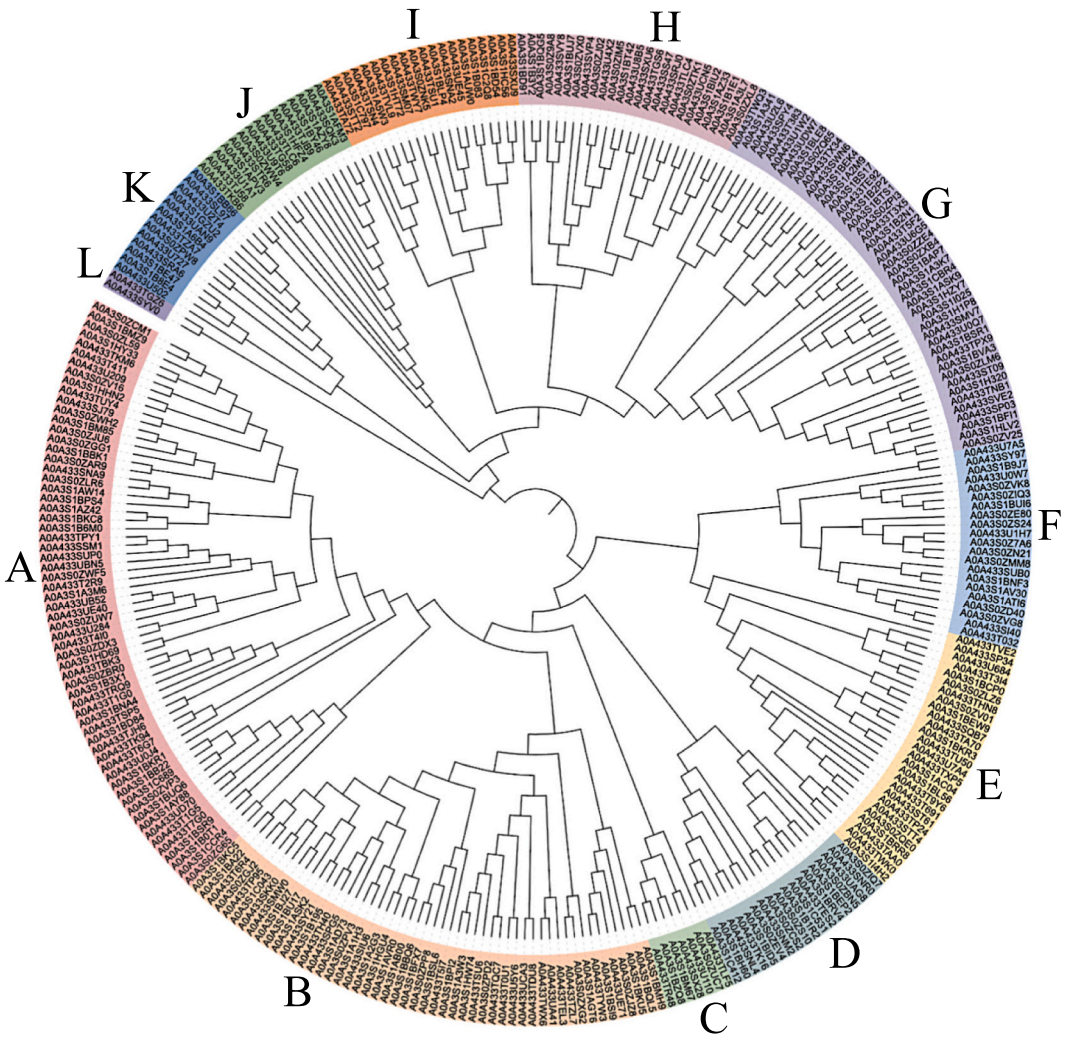

Fig. 3. Phylogenetic tree of proteins identified on the mucus of *Elysia crispata*. 12 phylogenetic subgroups have been identified. Groups are as follows: group K, protein folding activity; group A, protein catabolic process; group J, cytoskeleton organization; group E, signalling and transport; and group B, cellular amino acid metabolic process. For the remaining groups (C, D, F, G, H, I, and L) no primary biological functions could be assigned.

[85]. The main proteins from this family with potential biotechnological applications are glutathione transferases, which can be used for drug delivery systems [86].

The high abundance of hydrolases, particularly those acting on acid anhydrides, in the mucus of *E. crispata* suggests, once again, that this mucus may have antimicrobial properties, specifically through the action of lysozymes. Further investigation into the antimicrobial activity of *E. crispata* mucus could be a promising avenue for future research. Additionally, the presence of peptidases and transferases in the mucus suggests potential biotechnological applications for this material, which could be explored in further studies.

3.5. Comparative proteomics with gastropods species and marine animals

Tachapuripunya et al. [35] conducted a comparative proteomic analysis of the mucus from seven gastropod species to identify potential bioactive compounds. In their study, mucus samples were collected and analysed from commercially valuable gastropods, including *Achatina fulica, Pomacea canaliculata, Cryptozona siamensis, Semperula siamensis, Hemiplecta distincta, Cyclophorus fulguratus* and *Helix pomatia*. Gene Ontology analysis showed that proteins were involved in various processes, including biological processes, cellular processes, metabolic processes, biological regulation, localization, response to stimulus, signalling and cellular component organization or biogenesis were the most common. Comparing the results with our study, we can conclude that the mucus of *E. crispata* had biological functions similar to those of other gastropods. However, the main molecular function differed between species. The mucus of *E. crispata* had the main functions of hydrolase activity, GTPase activity and structural molecular activity, whereas the seven mentioned gastropods, had the main molecular functions of binding, catalytic activity and transporter activity.

Another study reported the proteome of mucus secretions from the sea anemone *Stichodactyla duerdeni*, which included actin, myosin, histone, and glyceraldehyde-3-phosphate dehydrogenase [87]. Similarly, a study conducted on mucus samples from another sea anemone, *Anthopleura dowii*, reported the presence of actin, myosin, elongation factors, and glyceraldehyde-3-phosphate [88]. These proteins were also found in the mucus of *E. crispata*. Jurado et al. [89] described the proteomic profile of the mucus of farmed fish *Sparus aurata*, which is primarily composed of structural proteins, such as actin and tubulin, and metabolic proteins. Other studies have also reported the presence of these molecules in mucus [90,91]. Based on these findings and previous studies, it can be concluded that actins, tubulins, histones, ATP synthase components and glycolytic enzymes are common proteins found in mucus samples. This consistency across studies suggests that these proteins are important components of mucus and potentially serve important roles in biological processes, such as motility, energy production, and cellular organization.

Table 2
The twenty most abundant proteins found in mucus samples of the sea slug *Elysia crispata,* with the accession number for the Uniprot database (Uniprot), the protein name, GO analysis (Cellular Component-CC; Molecular function-MF; Biological process-BP), mRNA accession number (Cai et al.[24]), number of peptides (#Pep), number of unique peptides (#UPep), molecular weight (MW (kDa)) and Protein percentage (Prot %).

Uniprot	Description	GO	mRNA	# UPep	# Pep	MW [kDa]	Prot %
A0A3S1HT72	Actin, cytoplasmic	No information	EGW08_006629 (Echl_07817)	8	21	41.9	20.92
A0A3S1BB22	VWFD domain-containing protein	No identification	EGW08_008094 (Echl_09288)	8	8	43.3	13.74
A0A433STR6	Tubulin beta chain	CC-microtubule MF-GTP binding, GTPase activity, structural constituent of cytoskeleton BP- microtubule-based process	EGW08_019542 (Echl_21065)	12	29	49.9	7.39
A0A3S0ZVG8	IF rod domain-containing protein	CC-Intermediate filament	EGW08_005366 (Echl_06438)	23	23	64.9	3.74
A0A433TQC7	Lysozyme	MF-lysozyme activity BP-peptidoglycan catabolic process	EGW08_008424 (Echl_09635)	4	4	16.1	3.59
A0A3S1AFM3	Tubulin alpha chain	CC- microtubule MF- GTP binding; GTPase activity, structural constituent of cytoskeleton BP- microtubule-based process	EGW08_001610 (Echl_02030)	1	19	50.1	3.44
A0A433TPX9	Carboxylesterase type B domain-containing protein	MF- Cholinesterase activity	EGW08_008662 (Echl_09883)	2	6	94.9	3.31
A0A3S1BU17	Uncharacterized protein (Fragment)	No identification	EGW08_000578 (Echl_00728)	4	4	49.4	2.21
A0A433TVL9	Actin, cytoplasmic	No information	EGW08_006628 (Echl_10433)	7	16	41.9	2.15
A0A3S1B195	Histone H4	CC- nucleosome, nucleus MF -DNA binding, protein heterodimerization activity BP-DNA-templated transcription, initiation	EGW08_022752 (Echl_24296)	9	9	28.9	2.12
A0A433TYW3	ATP synthase subunit beta	MF -Proton-transporting ATP synthase activity, rotational mechanism; Proton-transporting ATPase activity	EGW08_005486 (Echl_06569)	17	18	56.3	1.22
A0A3S0ZBN5	Inhibitor_I29 domain-containing protein	No identification	EGW08_020987 (Echl_19827)	3	3	13.0	1.14
A0A433U5Y6	Uncharacterized protein (Fragment)	CC-membrane MF -Hydrolase activity, acting on glycosyl bonds	EGW08_003106 (Echl_03785)	2	2	50.2	1.09
A0A3S1C531	Histone H2A	CC-nucleosome, nucleus MF- DNA binding, protein heterosimerization activity	EGW08_009066 (Echl_03850)	3	4	24.8	1.04
A0A3S1C412	14_3_3 domain-containing protein	No information	EGW08_009865 (Echl_05407)	11	13	29.2	0.998
A0A3S1A5W3	Actin, cytoplasmic	No information	EGW08_000781 (Echl_01084)	7	19	41.8	0.985
A0A3S1BLE8	Elongation factor 1-alpha	MF- GTP binding, GTPase activity, translation elongation factor activity	EGW08_008718 (Echl_09940)	8	8	50.3	0.935
A0A433SM07	Glyceraldehyde-3-phosphate dehydrogenase	MF-glyceraldehyde-3-phosphate dehydrogenase activity, NAD binding, NADP binding BP- glucose metabolic process, glycolytic process	EGW08_022033 (Echl_19895)	7	7	36.0	0.927
A0A3S1BSI9	ATP synthase subunit alpha	CC- proton-transporting ATP synthase complex MF- ATP binding; proton-transporting ATP synthase activity, rotational mechanism	EGW08_001746 (Echl_02216)	16	16	59.9	0.884
A0A3S1APV3	Histone H2B	CC- nucleosome, nucleus MF- DNA binding, protein heterosimerization activity	Not expressed	4	4	13.6	0.818

Based on this information and the various studies on mucus proteomics, it can be concluded that the protein composition and relative abundance are influenced by genetics, environmental factors and the type of mucus being analysed. For instance, studies that induce stress responses in animals show a different proteomic profile from the baseline level [31–33]. In this study, we did not detect any mucins, which may be due to the animals' "basal" level of stress, but also because gel-based proteomic approaches are not effective for the identification of mucin molecules [92]. Further investigations are necessary to examine the differences between "basal" and harmful stress conditions on the animal's mucus production and constitution. For example, when these animals are subjected to trauma, such as cuts, we have observed that they are coated in thick mucus that covers their entire body, as well as when they are subjected to extended periods of light stress.

It is reasonable to expect cytoskeleton proteins in mucus, as it is in close contact with skin cells and can be subject to skin desquamation. The high presence of energy-producing and motility proteins in mucus samples can be attributed to the two primary functions of mucus: protection and locomotion. The mucus acts as a protective layer for cells and tissues while also facilitating animal movement through their environment. The presence of these proteins indicates the potential role of mucus in these fundamental biological processes. However, further research is necessary to fully understand the specific roles of these proteins in mucus and their potential impact on animal physiology.

4. Conclusion

In this study, we described for the first time the identification and characterization of proteins found in the mucus secretions of the sea slug *Elysia crispata*. Using gel electrophoresis, we identified 11 major protein bands with molecular weights ranging from 75 to 11 kDa. The samples were then analysed for protein identification using HPLC-MS/MS, resulting in the identification of 306 proteins. The number of proteins identified was found to be lower than in some other studies, likely due to the limited number of entries for *E. crispata* in the Uniprot database. The most difficult aspect of this study, besides the lack of an annotated

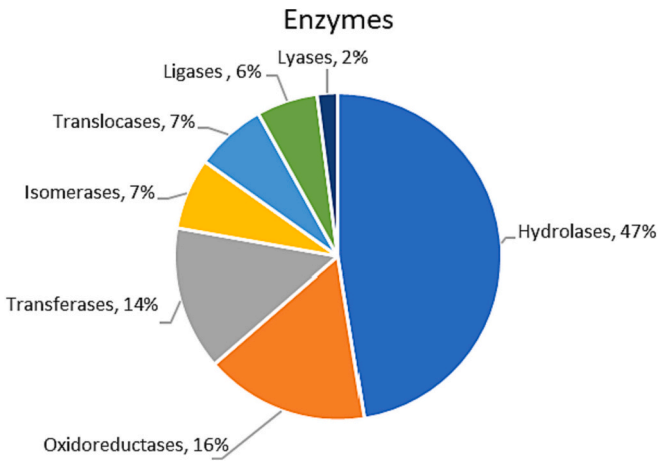

Fig. 4. The potential enzymatic activity of the proteins identified in the mucus of *Elysia crispata*. The classification and protein abundance have been determined as a percentage using the OmicsBox software. At the first level, seven main groups of enzymes have been identified.

Table 3
The potential enzymatic activity of the proteins identified in the mucus of *Elysia crispata* was classified into several subgroups. The classification and protein abundance has been determined as a percentage (%) using the OmicsBox software.

Enzyme family	Enzyme sub-family	%
Oxidoreductases	Oxidoreductases	2.41
	Acting on the CH-OH group of donors	4.82
	Acting on the aldehyde or oxo group of donors	3.61
	Acting on a peroxide as an acceptor	2.41
	Others	2.41
Transferases	Transferases	2.41
	Acyltransferases	1.20
	Glycosyltransferases	1.20
	Transferring nitrogenous groups	1.81
	Transferring phosphorus-containing groups	6.02
	Others	1.81
Hydrolases	Hydrolases	1.20
	Acting on ester bonds	3.61
	Glycosylases	4.22
	Acting on peptide bonds (peptidases)	4.82
	Acting on carbon-nitrogen bonds, other than peptide bonds	1.81
	Acting on acid anhydrides	31.3
Lyases	Carbon-carbon lyases	0.60
	Carbon-oxygen lyases	1.20
	Carbon-nitrogen lyases	0.60
Isomerases	Isomerases	0.60
	Intramolecular oxidoreductases	3.61
	Intramolecular transferases	0.60
	Racemases and epimerases	1.20
	Cis-trans-isomerases	1.20
Ligases	Ligases	3.01
	Forming carbon-oxygen bonds	1.81
	Forming carbon-sulfur bonds	0.60
	Forming carbon-nitrogen bonds	0.60
Translocases	Translocases	2.41
	Catalysing the translocation of hydrons	4.22
	Catalysing the translocation of inorganic cations	0.60

genome for our study model, was the mucus sampling technique in these animals. We evaluated the reproducibility of the mucus sampling technique and found no significant differences in protein abundance across the samples.

We proceeded with the functional annotation of the mucus proteome of *E. crispata*, using Gene Ontology (GO) and Omicsbox software. This analysis found that the mucus proteome of the sea slug is composed of proteins with various functions related to biological processes such as carbohydrate-derived metabolic processes and cytoskeletal organization. The study also identified a greater proportion of proteins with enzymatic activity in the mucus of *E. crispata*, which suggests the potential for biotechnological applications such as antimicrobial and antitumor activities. The analysis revealed the presence of the seven major enzyme types, with hydrolases accounting for 47% of all proteins with putative enzymatic activity. Within the hydrolase group, those acting on acid anhydrides comprised the majority of the mucus from this sea slug. The high abundance of hydrolases in the mucus of *E. crispata* may indicate the presence of antimicrobial properties.

Proteins common in mucus samples from various species including *E. crispata* include actins, tubulins, histones, ATP synthase components and glycolytic enzymes, which play important roles in biological processes such as motility, energy production, and cellular organization. The presence of these proteins and others found in the mucus of *E. crispata* suggests that mucus has important functions in protecting cells and tissues while facilitating animal movement through their environment. This study highlights the need for further research to fully understand the specific roles of these proteins in mucus and their potential impact on animal physiology, as well as the influence of genetics, environmental factors, and the type of mucus being analysed on the protein composition and relative abundance.

Funding

This work received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement no. 949880) (S.C.) and project AlgaValor, from the Portugal 2020 program (grant agreement n° POCI-01-0247-FEDER-035234; LISBOA-01-0247-FEDER-035234; ALG-01-0247-FEDER-035234) (P.D.). We acknowledge support from Fundação para a Ciência e a Tecnologia (FCT/MCTES) through individual grants no. 2020.08672.BD (D.L.), 2020.03278.CEECIND (S.C.) and CEECIND/01434/2018 (P.C.), as well as financial support to LAQV/REQUIMTE (UIDP/50006/2020 + UIDB/50006/2020), CESAM (UIDP/50017/2020 + UIDB/50017/2020 + LA/P/0094/2020) and RNEM - Portuguese Mass Spectrometry Network (LISBOA-01-0145-FEDER-402-022125).

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used CHATGPT v4 in order to improve the quality and clarity of the English language. After using this tool, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

CRediT authorship contribution statement

Diana Lopes: Data curation, Investigation, Methodology, Validation, Writing – original draft, Formal analysis. **Susana S. Aveiro:** Formal analysis, Investigation, Validation. **Sónia Cruz:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Paulo Cartaxana:** Conceptualization, Funding acquisition, Methodology, Project administration, Writing – review & editing. **Pedro Domingues:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no financial or other possible competing interests.

Data availability

The mass spectrometry proteomics data have been deposited to the

ProteomeXchange Consortium via the PRIDE [93] partner repository (http://proteomecentral.proteomexchange.org) with the dataset identifier PXD042643 and https://doi.org/10.6019/PXD042643

Acknowledgements

The authors thank Miguel Pereira for assistance in the statistical analysis and Anthony Moreira for assistance with the staining microscopy methodology.

Parts of the Graphical Abstract were drawn by using pictures from Servier Medical Art. Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2024.105087.

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