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Chapter 31

Characterization of Soluble Cell-Free Coelomic Fluid Proteome from the Starfish *Marthasterias glacialis*

Laidson Paes Gomes, Catarina Gouveia e Silva, Jean-Charles Gaillard, Jean Armengaud, and Ana Varela Coelho

Abstract

Proteomics combined to advanced bioinformatics tools is acquiring a pivotal role in the comprehensive understanding of living organism's biology, in particular for non-model organisms, which includes most marine and aquatic invertebrates. Depicting of protein composition in a whole organ/organism followed by their assembling in functional protein association networks promotes the understanding of key biological processes. Here, we provide a detailed description of the extraction procedure of cell-free coelomic fluid soluble proteins and the characterization of the proteome of the starfish *Marthasterias glacialis*. Due to coelomic fluid richness in glycoproteins, which complicates protein identification, extracts of soluble proteins are deglycosylated prior to tandem mass spectrometry. This experimental approach is useful at improving knowledge on the coelomic fluid physiological role and deciphering its involvement in regeneration of starfish body parts when comparing different regeneration conditions.

Key words Starfish, *Marthasterias glacialis*, Coelomic fluid, Proteomics, Tandem mass spectrometry

1 Introduction

Proteomics is the study of multiple protein systems with focus on the interplay of multiple, distinct proteins, and their roles as part of a larger system or network [1]. Mass spectrometry (MS), coupled to liquid chromatography (LC–MS), is the most used well-established methodology for proteome investigations [2]. A mass spectrometer measures the mass/charge ratio (*m/z*) of generated peptide molecular ions and their relative abundances allowing to characterize, identify, and quantify peptides and proteins. Protein extracts rich in glycoproteins can be enzymatically deglycosylated prior to LC-MS analysis to extend the number of identified proteins [3]. To make sense of the extensive sets of identified proteins by proteomics, a functional analysis is required. It tends to organize the identified proteins in biochemical, cellular, biological, and disease-related categories according to the process that they are

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associated with. These different steps are hampered in non-model organisms due to the lack of biological knowledge and complete genome information.

Regenerative potential is expressed to a maximum extent in echinoderms. Starfish are capable of reconstructing external appendages and internal organs often subjected to amputation, self-induced or traumatic, rapidly followed by complete successful re-growth of lost parts. *Marthasterias glacialis* (Linné 1758) is a fairly common asteroid echinoderm widely distributed throughout the northern Europe; it is often found on the Atlantic coast of central/northern Portugal due to its preference for glacial waters. This spiny starfish, which surface is covered with thorns, can be found in waters up to 200 m in a variety of habitats from muddy, protected locations to rock exposed waves [4]. *M. glacialis* normally reaches 25–30 cm in diameter, has five arms, each having three rows of thorns, and differs in color from dark brown to greenish gray. It is a voracious predator feeding on various animals, dead or alive, such as mollusks, fishes, crustaceans, or other echinoderms [5]. Starting from only one fifth of the central disc, *M. glacialis* can survive and regenerate a new individual [4]. However, its regeneration process is slow and complex. Its arm tip regeneration, or even of an entire arm lost by autotomy, can take from a couple of weeks to several months [4]. This species can be seen as an important model for the study of regeneration due to 70% genome similarity with humans [4], its extraordinary regeneration ability [6], and its easy collection from the wild and maintenance in aquaria.

The coelomic fluid circulates the water vascular system, an internal network composed of channels that contacts their internal organs. The liquid part of the coelomic fluid consists of seawater and is extremely rich in secreted molecules. This fluid is responsible for the transport of circulating cells (coelomocytes), nutrients, and metabolites bathing the internal organs [7]. Major coelomocyte morphotypes have the primary function of mediating immune responses, being able to recognize and neutralize foreign material [8, 9]. Humoral responses on echinoderms are represented by a great variety of molecules, like lectins, perforins, and cytokines, secreted by coelomocytes or surrounding tissues, and promoting cell migration, agglutination, and healing [9]. Recently, the *Asterias rubens* coelomocyte-free coelomic fluid proteome was characterized by LC-MALDI tandem mass spectrometry, identifying 91 proteins [10]. The most represented functional categories were pattern recognition receptors and peptidase inhibitors. Proteins known to be involved in the process of sea cucumber intestinal regeneration, such as ependymin, β-microseminoprotein, serum amyloid A and avidin-like proteins, have also been identified. Proteome characterization of cell-free coelomic fluid soluble proteins suggests that this fluid plays an important role in cell signaling,

transport, and responses to injury in starfish, constituting a relevant tissue to be studied to deeply elucidate the molecular processes associated with starfish organ regeneration. The protocol presented here below can be easily applied to other invertebrate fluids and to quantitative differential proteomics studies involving regeneration or other physiological challenges.

Figure 1 summarizes the shotgun proteomics experimental workflow for the characterization of the soluble proteome of cell-free coelomic fluid (CFF) from *M. glacialis*. In order to extend the number of identified proteins, half of the precipitated protein extract is deglycosylated with Peptide-N-Glycosidase F (PNGase F). PNGase F enzymatic treatment removes the N-linked oligosaccharides from glycoproteins since it cleaves between the innermost N-acetylglucosamine (GlcNAc) and asparagine residues of high mannose, hybrid, and complex oligosaccharides [3]. Since this step can reduce the sensitivity of the analytical procedure, the non-deglycosylated extract is also assayed. Proteins are in-gel digested with trypsin previous to identification by LC-MSMS analysis. Functional analysis of the identified proteins, including prediction of N-glycosylation sites [11], is used to allow a comprehensive description of the metabolic and biological processes occurring in this tissue.

2 Materials

Use bidistilled water and room temperature unless otherwise stated.

2.1 Sample Preparation

1. Adult wild *Marthasterias glacialis* (*see* **Notes 1** and **2**).
2. Venipuncture needle (e.g., Venofix Safety, B Braun).
3. Protein precipitation solution: 40% (w/v) trichloroacetic acid, 0.028% (v/v) β-mercaptoethanol.
4. −20 °C cold acetone pro-analysis grade.
5. 50 mM ammonium bicarbonate (AB).
6. Protein solubilization solution: 6 M urea, 50 mM AB.
7. Total protein quantification kit (e.g., BCA assay).
8. Peptide-N-Glycosidase F (PNGase F) Kit (New England Biolabs, Fisher Scientific): containing 500 mM sodium phosphate and 10% (w/v) NP-40.

2.2 Denaturing Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Resolution gel buffer: 1.5 M Tris–HCl, pH 8.8, 0.1% (w/v) sodium dodecyl sulfate (SDS).
2. Stacking gel buffer: 0.5 M Tris–HCl, pH 6.8, 0.1% (w/v) SDS.

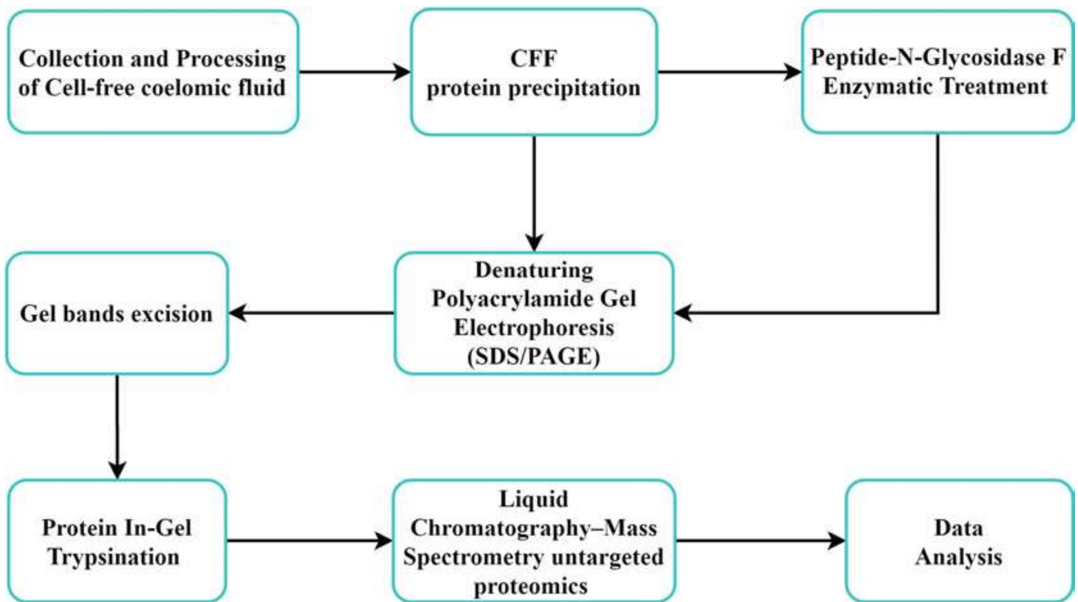

Fig. 1 Experimental workflow for the characterization of CFF soluble proteome from *M. glacialis*. Each step of this protocol is described in text boxes and the workflow follows the sequence of events indicated by the arrows

3. 40% (w/v) acrylamide/bis-acrylamide (crosslinker ratio 29:1): 38.67% (w/v) acrylamide, 1.33% (w/v) bis-acrylamide.
4. 10% (w/v) SDS.
5. 10% (w/v) ammonium persulfate (APS).
6. Tetramethylethylenediamine (TEMED).
7. 12.5% (w/v) acrylamide resolution gel solution: 4.2 mL water, 2.5 mL resolution gel buffer, 3.1 mL 40% (w/v) acrylamide/ bis-acrylamide, 100 μL 10% (w/v) SDS, 100 μL 10% (w/v) APS, 5 μL of TEMED.
8. 5% (w/v) Acrylamide resolution gel solution: 3.18 mL water, 1.26 mL resolution gel buffer, 500 μL 40% (w/v) acrylamide/ bis-acrylamide, 50 μL 10% (w/v) SDS, 50 μL 10% (w/v) APS, 5 μL TEMED.
9. 4× sample loading buffer: 0.25 M Tris–HCl pH 6.8, 10% (v/v) β-mercaptoethanol, 8% (w/v) SDS, 0.02% (w/v) bromophenol blue, 30% (v/v) glycerol.
10. 1× SDS-PAGE running buffer: 25 mM Tris–HCl, 192 mM glycine, 0.1% (w/v) SDS.
11. Coomassie staining solution: 0.05% (w/v) Coomassie brilliant blue R-250, 50% (v/v) ethanol, 10% (v/v) acetic acid.
12. Destaining solution: 30% (v/v) ethanol, 10% (v/v) acetic acid.

13. Polyacrylamide gel electrophoresis system (e.g., Mini-PROTEAN® Tetra system, Bio-Rad).
14. Polyacrylamide gel electrophoresis system power supply (e.g., Amersham BioSciences Electrophoresis EPS 301).

2.3 Protein In-Gel Trypsination

1. Trypsin solution: 0.01 μg/μL mass spectrometry grade trypsin (e.g., Trypsin Gold, Promega) in 0.01% (v/v) trifluoroacetic acid.
2. Enhancer of trypsin enzymatic performance: 0.01% (v/v) ProteaseMax™ in AB.
3. 50% (v/v) methanol in AB.
4. Acetonitrile pro-analysis grade (ACN).
5. 50% (v/v) ACN in AB.
6. 25 mM DTT in AB.
7. 55 mM iodoacetamide in AB.
8. 5% (v/v) trifluoroacetic acid.

2.4 Untargeted Proteomics and Data Analysis

1. Nanoscale liquid chromatography system (e.g., nano LC system, UltiMate 3000 RSLC, Dionex).
2. High-resolution tandem mass spectrometer incorporating an ultra-high-field Orbitrap analyzer (e.g., Q Exactive HF Hybrid Quadrupole-Orbitrap™, ThermoFisher Scientific).
3. Desalt reversed-phase (RP) C18 trapping column (e.g., Acclaim PepMap 100, 5 μm, 100 Å, 300 μm id × 5 mm, ThermoFisher Scientific).
4. RP C18 analytical column (e.g., Acclaim PepMap 100, 3 μm, 100 Å, 75 μm id × 500 mm, ThermoFisher Scientific).
5. Solvent A: 0.1% (v/v) formic acid (FA).
6. Solvent B: 80% (v/v) ACN, 0.1% (v/v) FA.
7. Feature extraction software: ProteomeDiscoverer 2.3 (Thermo) for .RAW file to .MGF file conversion.
8. Protein identification software: MASCOT 2.5.1 (Matrix Science).
9. Protein sequences database (*see* **Note 3**).
10. Protein similarity search tool: BLAST [12].
11. Pathway Database: KEGG [13].
12. Protein molecular interaction open source software STRING [14].
13. Prediction of N-glycosylation sites in proteins tool: NetNGlyc [11].

3 Methods

3.1 Cell-Free Coelomic Fluid Collection and Protein Precipitation

1. Transfer a wild starfish onto the bench (*see* **Note 4**).
2. Puncture the starfish arm tip with a Venipuncture needle.
3. Hold the animal with the punctured arm hanging.
4. Place a 15-mL tube below the arm with the Venipuncture capillary inside it.
5. Collect 15 mL of the coelomic fluid by gravity into the tube.
6. Centrifugate the sample at 1000 rcf for 5 min at 4 °C (*see* **Note 5**).
7. Transfer the supernatant, the Cell-Free Coelomic Fluid (CFF), to a new 15-mL tube.
8. Discard the circulant cells (pellet).
9. Transfer 7.5 mL of CFF into a 15-mL tube.
10. Add to both the tubes 7.5 mL of protein precipitation solution (*see* **Note 6**).
11. Incubate both the tubes overnight at 4 °C.
12. Centrifugate the tubes at 10,000 rcf for 30 min at 4 °C.
13. Discard the supernatant.
14. Add 10 mL of ice-cold acetone to one of the tubes.
15. Resuspend the protein pellets by pipetting up and down 20 times.
16. Transfer the resuspended protein pellet to the second tube.
17. Repeat **steps 12–15** to remove the residual trichloroacetic acid and β-mercaptoethanol.
18. Dry the protein pellet under a N_2 flux.
19. Add 20 μL of protein solubilization solution.
20. Vortex the tube 30 s to solubilize the pellet.
21. Repeat **steps 19** and **20** until complete pellet solubilization (*see* **Note** 7).
22. Measure the total protein concentration following the kit manufacturer's instructions.

3.2 Peptide-N-Glycosidase F Enzymatic Treatment and SDS-PAGE Sample Preparation

1. Transfer 40 μg of the CFF protein extract to a 1.5-mL tube.
2. Denature the proteins by heating at 99 °C for 10 min (*see* **Note 8**).
3. Add 8 μL of 500 mM sodium phosphate, 8 μL of 10% NP-40, 4 μL of PNGase F.
4. Adjust the final volume of the mixture to 82 μL adding ultrapure water.

5. Incubate the reaction mixture at 37 °C for 1.5 h.
6. Concentrate four times the deglycosylated protein extract in a vacuum centrifuge.
7. Transfer 40 μg of the CFF protein extract to a 1.5-mL tube.
8. Add 5 μL of the 4× sample loading buffer to each protein sample, either deglycosylated or not (*see* **Note 9**).
9. Vortex the sample mixture for 30 s.
10. Heat the sample mixture for 5 min at 99 °C.

3.3 Denaturing Polyacrylamide Gel Electrophoresis (SDS-PAGE): Gel Casting and Sample Running

1. Cast the gel using the polyacrylamide gel electrophoresis system and following the manufacturer's instructions (Fig. 2).
2. Assemble the gel cassette using 7-cm glass plates and 1.0-mm spacers.
3. Fill up the space between the glass plates to below the bottom of the comb position with the acrylamide resolution gel solution.
4. Add a small layer of water to the top of the gel (*see* **Note 10**).
5. Leave it 30 min till the completion of the acrylamide polymerization process.
6. Remove the water layer from the top of the resolution gel.
7. Add the acrylamide stacking gel solution until the inter plates space in the casting chamber is completely full.
8. Insert the comb between the plates in the upper part of the gel cassette.
9. Leave the casting system for 30 min till the completion of the acrylamide polymerization process.
10. Remove the gel cassette from the casting stand.
11. Place the gel cassette in the electrode assembly together with a dummy gel cassette.
12. Remove the comb from the gel cassette.
13. Pour 1× SDS-PAGE running buffer into the opening of the casting frame between the gel cassettes, until it fills the wells of the gel.
14. Fill the region outside the frame with 1× SDS-PAGE running buffer.
15. Load 40 μg of each non-digested and PNGase F digested CFF protein samples into different gel well.
16. Conduct the protein separation by electrophoresis at 200 V, 60 mA (for our Amersham BioSciences Electrophoresis Power Supply EPS 301) until the samples migrate 2 cm (*see* **Note 11**).

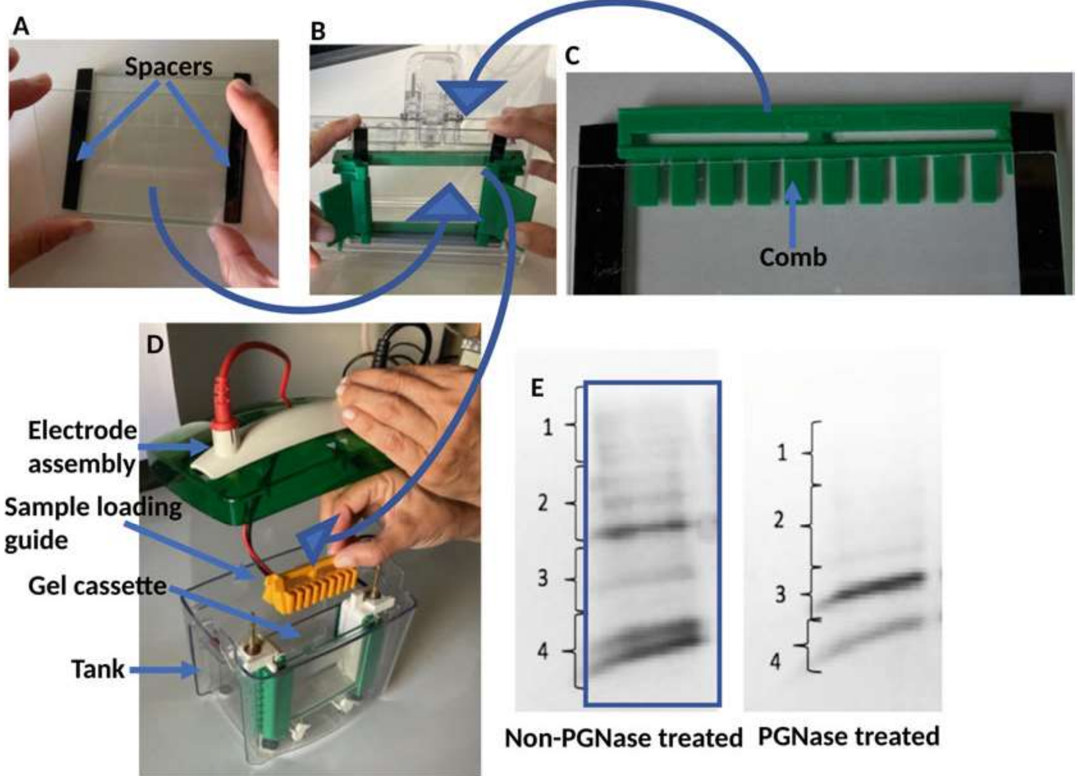

Fig. 2 Polyacrylamide gel electrophoresis: gel casting and sample running. (**a**, **b**) Assembling the gel cassette using two glass plates and two spacers. (**c**) The comb is introduced between the two glass plates after pouring the acrylamide stacking gel solution. (**d**) The gel cassette with the polymerized acrylamide (polyacrylamide) is included in the electrode assembly before introducing it inside the tank of the electrophoresis system. (**e**) Protein bands stained with Coomassie Blue after 2 cm migration of the non-deglycosylated and deglycosylated CFF total protein extract. The rectangle delimits the region of the gel to be excised and divided in four strips, here defined by the square brackets, before in-gel trypsinization

3.4 Coomassie Blue Gel Staining

1. Disassemble the polyacrylamide gel electrophoresis system.
2. Separate the glasses of the gel cassette.
3. Transfer the gel into a glass recipient and wash it with ultrapure water (*see* **Note 12**).
4. Cover the gel with the Coomassie staining solution.
5. Leave it overnight.
6. Remove the staining solution.
7. Add the destaining solution to the gel.
8. Renew the destaining solution until removing the blue background (Fig. 2c).
9. Substitute the destaining solution by water.
10. Keep the gel at 4 °C to avoid dehydration.

3.5 Protein In-Gel Trypsination

1. Cut each gel band by the limit of gel wells, between the front of migration and the well bottom in 0.5 cm height bands with a scalpel.
2. Transfer the bands to separate 1.5-mL tubes.
3. Add 200 μL of 50% (v/v) methanol in AB.
4. Vortex for 1 min at 500 rpm.
5. Repeat **steps 3** and **4** to destain the gel bands.
6. Replace the solution with 200 μL 50% (v/v) ACN in AB.
7. Vortex for 5 min at 500 rpm.
8. Replace the solution with 100% acetonitrile.
9. Vortex for 1 min at 500 rpm.
10. Remove the supernatant.
11. Dry the gel bands in a vacuum centrifuge.
12. Add 100 μL of 25 mM DTT in 50 mM AB.
13. Incubate for 10 min at 56 °C under 500 rpm agitation in a ThermoMixer® to rehydrate the gel and reduce the proteins persulfate bonds.
14. Add 100 μL of 55 mM iodoacetamide in 50 mM AB.
15. Incubate for 10 min in the dark to alkylate the proteins thiol groups.
16. Wash twice the gel bands with water as in **steps 9** and **10**.
17. Dehydrate the gel bands as described in **step 11**.
18. Incubate the dried in-gel digests with 40 μL 0.01 μg/μL trypsin in 0.01% (v/v) trifluoroacetic acid for 15 min on ice.
19. Discard the supernatant.
20. Add 70 μL of the enhancer of trypsin enzymatic performance solution.
21. Incubate for 1 h at 50 °C.
22. Stop the proteolysis by adding 5 μL of 5% (v/v) trifluoroacetic acid (*see* **Note 13**).

3.6 Liquid Chromatography–Mass Spectrometry Untargeted Proteomics Analysis

1. Load 3 μL of each tryptic peptide mixture to be online desalted on the RP C18 trapping column.
2. Resolve the tryptic peptides on the RP C18 analytical column at a flow rate of 200 nL/min with a 90 min gradient of 4–25% of solvent A in 75 min and 25–40% of solvent B in 15 min (*see* **Notes 14** and **15**).
3. The mass spectrometer is operated in data-dependent method consisting in a scan cycle initiated with a full scan of peptide ions, followed by selection of the precursor molecular ion, high energy collisional dissociation, and MS/MS scans on the

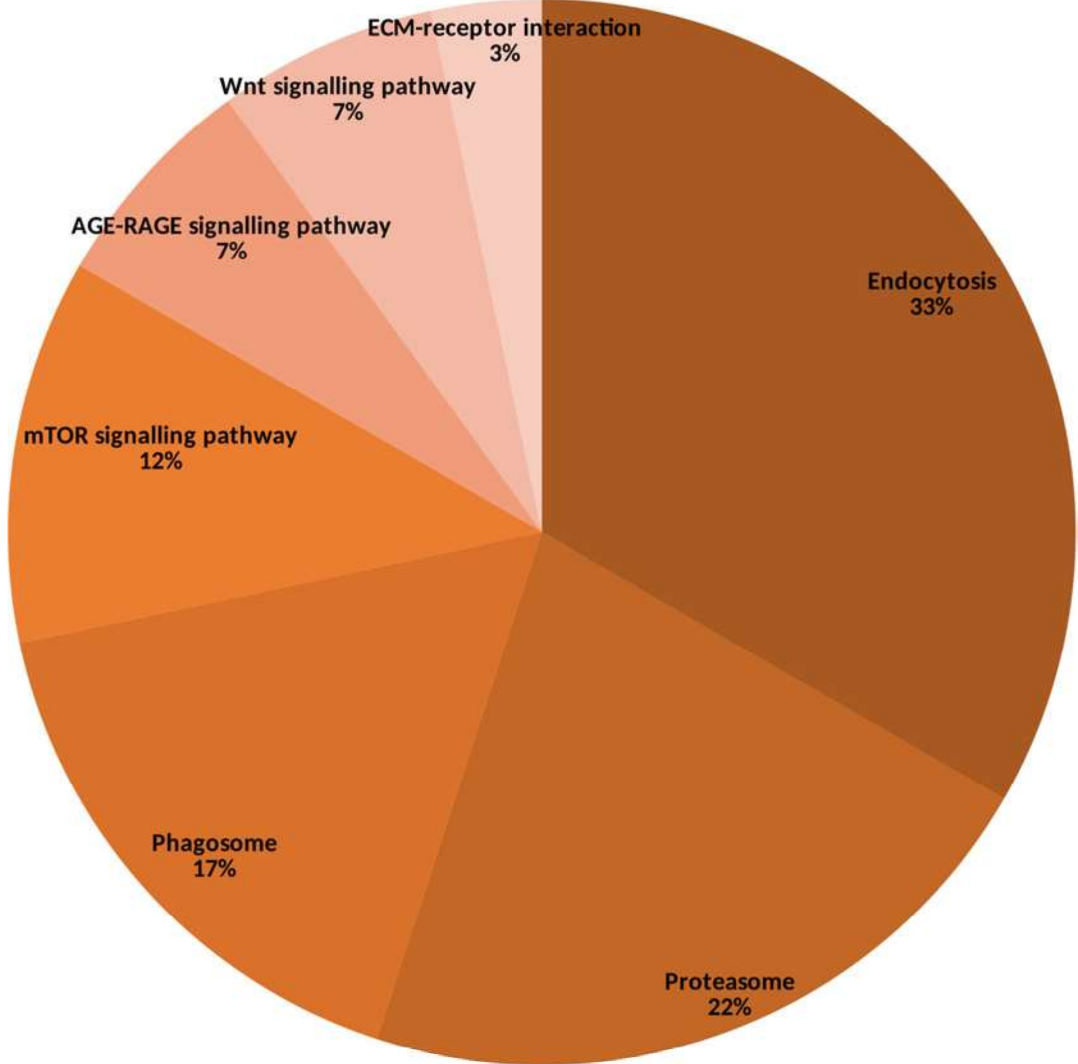

Fig. 3 Pie chart illustrating chosen relevant KEGG pathways from STRING. A total of 51proteins were classified in seven biological processes other than carbon metabolism

20 most abundant precursor ions. Full scan mass spectra are acquired from *m/z* 350–1500 with a resolution of 60,000. Each MS/MS scan is acquired with a threshold intensity of 83,000, on potential charge states of 2+ and 3+ after ion selection performed with a dynamic exclusion of 10 s, maximum IT of 60 ms and an *m/z* isolation window of 2.

4. MS/MS spectra at a resolution of 15,000 are search against the established echinoderm assembled protein database using MASCOT 2.5.1 software. The peptide matches with a MASCOT peptide score below a *p*-value of 0.05 were filtered and assigned to proteins.

5. Additionally, the following parameters are used for MS/MS spectra assignation: full-trypsin specificity, maximum of two missed cleavages, mass tolerances of 5 ppm on the parent ion, fixed modification of carbamidomethyl cysteine (+57.0215), and oxidized methionine (+15.9949) and deamidated asparagine/glutamine (NQ) (0.9840) as dynamic modifications. The mass tolerance for the MS scans is 0.02 Da (*see* **Note 16**).
6. Proteins are validated with the MASCOT decoy search mode, considering a valid protein when at least two different specific peptides are detected in the best sample with a false discovery rate below 1% (*see* **Note 17**).
7. Abundance of each protein is calculated with the sum of spectra assigned to the corresponding peptides (*see* **Note 18**).
8. Functional analysis of identified proteins is performed with the STRING web interface, using *Strongylocentrotus purpuratus* as the homologous organism and a minimum required interaction score with high confidence (0.7).
9. For the proteins not assigned to KEGG pathway classifications in STRING, a protein Blast sequence homology search is performed, using Echinodermata as a taxonomy restriction. Any protein with less than 50% query cover and 70% identity or more than 1×10^{-20} e-value is dismissed, and the best match is chosen (*see* **Note 19**, Fig. 3).
10. Some proteins are identified only in the deglycosylated samples with an asparagine residue to aspartic acid modification, and so with potentially glycosylated peptides, suggesting these are originally glycoproteins. Use NetNGlyc [11] to predict the eventual existence of N-glycosylation sites, thus further validating the results interpretation (*see* **Note 20**).

4 Notes

1. Collect wild starfish at low tides on suitable rocky beaches from the European Atlantic Coast. We collect our *Marthasterias glacialis* on the west coast of Portugal (38.701850° N, 9.392015° W).
2. *Marthasterias glacialis* specimens can be kept for 1 year in open-circuit tanks with recirculating sea water, at 15 °C and 33‰ and fed ad libitum with a diet of mussels.
3. If a homology search has to be performed, namely due to the unavailability of a quality genome sequence for the species under study or for an homologous organism, a compilation of the available proteomes for the close taxonomic related species should be done and used as the protein search database. Since *M. glacialis* genome sequence is not available, protein

identification was performed using the protein sequences available from other echinoderm specimens. This database was constructed compiling protein data from several databases, such as NCBI (https://www.ncbi.nlm.nih.gov/protein/), UniProt (https://www.uniprot.org/), and Echinobase (https://www.echinobase.org/entry/).

4. In order to avoid contamination of coelomic fluid samples with human proteins, in particular keratins, it is strongly advised to wear powder-free nitrile gloves till ending the protein trypsination step.

5. Low speed centrifugation avoids the coelomocytes lysis, that will cause a contamination of CFF with the circulating cell proteins.

6. Further details on the mechanism of protein precipitation by TCA can be found in reference [15].

7. The necessary volume of protein solubilization solution will be dependent on the amount of precipitated protein. In order to keep a high protein concentration, the minimum amount of the protein solubilization solution should be used. Although, it is very important to assure that the whole pellet was solubilized before proceed to the quantification step.

8. PNGase F (Peptide-N-Glycosidase F) treatments are performed using the PNGase F Kit (New England Biolabs, Fisher Scientific) following the manufacturer's instructions with some adjustments. In order to maximize the amount of protein to be digested, the addition of the denaturant solution is substituted by heat denaturation. This procedure adjustment do not make significant changes to the efficiency of protein deglycosylation.

9. Description of the role of 4× sample loading buffer compounds follows. SDS is an anionic detergent that promotes protein denaturation by binding to the vast majority of proteins and breaking the majority of the non-covalent interactions. The SDS-desaturated protein bound formed give every protein the same charge-to-mass ratio. Additionally, and since gels have sieving properties, mobility becomes a parameter for protein discrimination as a function of molecular mass [16]. 2-Mercaptoethanol is added to the protein sample to break up persulfate bonds and to prevent oxidation of cysteines. The denaturation process is extended by overheating the sample at 100 °C. Bromophenol blue is a dye used to help visualizing protein sample load in the well and tracking its progress through the gel. Glycerol has a higher mass density than water, causing the sample to fall to the bottom of the well, avoiding overflow and consequent loss of the sample. 4× sample loading buffer is added in a proportion 1:4 (v:v) to the total protein extract.

10. The water layer will prevent the diffusion of oxygen that acts as an inhibitor of acrylamide polymerization.
11. This SDS-PAGE step allows to clean the protein sample from eventual interferents of the LC-MS assay. Additionally, it is used to obtain less complex protein samples to extend the number of identified proteins.
12. In order to avoid contamination with non-CFF proteins, dedicated plastic containers or glass containers should be used in this step.
13. The final volume of the tryptic peptide mixture is 50 μL.
14. Conditions and parameters are provided for the separation of tryptic peptide mixtures in an UltiMate 3000 RSLCnano LC system (Dionex) and analyzed with a Q Exactive HF high-resolution tandem mass spectrometer (ThermoFisher Scientific), incorporating an ultra-high-field Orbitrap analyzer. Sample running conditions and parameters could need adjustments if another high-resolution LC-MS/MS system is used for this assay.
15. The average total protein concentration in the CFF is 9 ± 3 μg/mL. Considering its minimum concentration (6 μg/mL), the volume of collected coelomic fluid (15 mL) will allow to assay two replicates by SDS-PAGE (40 μg/each run that generates 50 μL of tryptic peptide mixture/replicate) and a total of 15 LC-MSMS assays (3 μL/each run).
16. The specified amino acid modifications are originated during the in-gel protein trypsinization protocol depicted in Subheading 3.6. Fixed modifications are expected to affect all cysteine residues, while dynamic modifications can happen in some methionine, asparagine, or glutamine residues. The carbamidomethylation of cysteine results from the alkylation reaction promoted by iodoacetamide.
17. Using the herein described workflow based on shotgun proteomics, it was possible to identify in total 1717 proteins of which 380 were certified with at least two non-ambiguous peptides.
18. The abundance of each protein is not used for proteome characterization, but for differential proteomics studies aiming to compare the levels of each detected protein in diverse experimental conditions, namely for regeneration studies. Multi- and univariate analyses should be performed to determine the discriminant proteins among experimental conditions.
19. From the 380 proteins identified in *M. glacialis* coelomic fluid using the herein described protocol, 51 proteins were classified into pathways not included in the central metabolism (endocytosis, proteasome, phagosome, extracellular matrix-receptor

interaction, mTOR, AGE-RAGE, and Wnt signaling pathways).

20. PNGase F removes the internal *N*-linked oligosaccharides from glycoproteins. This enzyme catalyzes the cleavage between N-acetyl-D-glucosamine and asparagine residues. The asparagine residue from which the oligosaccharide is removed is deaminated to aspartic acid. For *M. glacialis* coelomic fluid 43 proteins were only detected after PNGase F treatment and with an asparagine residue to aspartic acid modification. Thirty-seven out of these proteins had glycosylation site predictions, and 31 had more than one glycosylation site predictions.

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