Proteomics of fish white muscle and Western blotting to detect putative allergens

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Abstract

A detailed workflow is provided for preparation from teleost fish white muscle of extracts for proteomics analysis. The protocol generates samples that can be analysed by SWATH (Sequential Window data independent Acquisition of the Total High-resolution-Mass Spectra), a modern MS based quantitative label free technology. The main steps for the extraction of three independent protein fractions, i) soluble sarcoplasmic, ii) soluble myofibrillar and iii) insoluble material, from fish white muscle are detailed. Coupled to the protein extraction protocol a Western blotting approach is outlined for detection of common fish allergens, in this case ß-parvalbumin, in the white muscle sarcoplasmic protein fraction.

Key words: Allergen, ß-parvalbumin, European sea bass, protein extraction, Proteomics, Western blot

1. Introduction

Fresh fish consumption has significant "health benefits" since they are a source of high-quality protein, omega-3 fatty acids and trace nutrients. The white muscle of fish is the main commercial target of the fishing industry, since it is preferred for human consumption. High-throughput technologies for monitoring quality and safety of sea food products are needed and proteomics technology is a promising strategy to fill this gap. Proteomics is used in a panoply of applications under the catchy term "foodomics". In the case of sea food products, the main applications of proteomics techniques are for species authentication, monitoring of fish spoilage and quality during storage and processing and allergen detection [1, 2, 3]. The latter is of increasing importance since fish are one of the eight most common causes of allergy and there is an ambition to increase fish production and consumption to meet global protein needs [4]. Existing proteomics studies applied to sea food products are mainly based on conventional technologies and have identified a relatively small number of proteins. The application of high throughput quantitative methodologies such as SWATH-MS (Sequential Window data independent Acquisition of the Total High-resolution-Mass Spectra), a quantitative label free technology can accelerate discovery and progress [5, 6].

The three main steps in the proteomics workflow are, i) protein extraction from samples, ii) protein/peptide separation and iii) protein identification/quantification. The first step is crucial and protein extraction should yield good quality proteins that are representative of the sample. This means methods should be sample-specific with the view of extracting proteins and peptides suitable for mass spectrometry. Three main protein fractions can be extracted from fish white muscle: 1 - sarcoplasmic (around 30% of muscle protein, low salt soluble, enriched in enzymes); 2 - myofibrillar (around 60% of muscle protein, high salt soluble, enriched in structural proteins); and 3 -

stromal (less abundant, enriched in connective proteins). In the following section a detailed workflow for protein extraction from teleost fish white muscle that is compatible with SWATH proteomics analysis is outlined, using the European sea bass (*Dicentrarchus labrax*) as the example [7, 8]. The protocol provides the main steps for efficient extraction of the three main protein fractions. The method integrates mechanical disruption and successive protein fractionation steps to solubilize proteins by exploiting their biochemical properties. These kinds of approaches have been reported in a relatively restricted literature with a variable scope for several commercial fish species [9, 10, 11, 12, 13]. The method presented herein was successfully used for SWATH analysis of the sarcoplasmic and myofibrillar fractions of European sea bass white muscle and is compatible for use with white muscle from other fish species [7, 8].

Fish consumption is one of the eight most common causes of allergy. This is due to an IgE-mediated immune reaction, in a small proportion (<1% worldwide) [14] of people that consume fish, and it can be triggered by proteins such as parvalbumin [2, 14]. Parvalbumins are muscle sarcoplasmic calcium-binding proteins, that are resistant to high temperatures, denaturing agents, and proteolytic activity. The detection of fish parvalbumins is challenging compared to other food allergens. This may be due to isoform diversity and high biochemical and immunological variability, despite generally good amino acid sequence conservation of parvalbumin from different fish species [15, 14].

Using β-parvalbumin as an example of an allergen, we describe a second protocol based on Western blotting using European sea bass extracts of the white muscle sarcoplasmic protein fraction. Western blotting is a powerful and sensitive immunochemical technique developed in the seventies [16]. It is based on the use of specific antisera to

71 detect proteins in a complex sample after separation by sodium dodecyl sulfate-
72 polyacrylamide gel electrophoresis (SDS-PAGE), [17] and transfer and immobilization
73 on nitrocellulose membranes [18, 19].

74 2. Materials

75 Prepare and store all reagents at room temperature (unless indicated otherwise). Use
76 ultrapure water (UP water, Milli-Q® Type 1 or equivalent), unless indicated otherwise.
77 Diligently follow regulations when disposing of waste materials.

78 2.1 Global protein extraction from fish white muscle

79 To avoid contamination in MS proteomics analysis special care should be taken during
80 protein sample preparation. Precautions include, the use of gloves and a lab coat, use
81 of clean material, low protein binding tubes, and highly pure reagents. All protein
82 preparation steps, when possible, should be done in a laminar flow cabinet or failing
83 this in a clean environment.

84 2.1.1 Protein extraction

85 1. Hammer, mortar, pestle and aluminum foil.
86 2. Liquid nitrogen (*see* **Note 1**).
87 3. 2 mL micro tubes with cap, compatible with a tissue homogenizer in use
88 (indicated below in **11**).
89 4. Glass beads (0.5 mm).
90 5. Micro spoon spatula (5 x 7 mm)
91 6. 6 M HCl (hydrochloric acid): Weigh 21.84 g of HCl and add dropwise to 100
92 mL of UP water and store at room temperature.

7. 1 M DTT: Weigh 1.54 g DTT (1,4-Dithiothreitol) and dissolve in 10 mL of UP water. Create aliquots of 1 mL and store at -20 ºC.
8. B1 - Sarcoplasmic protein extraction buffer: 10 mM Tris-HCl, pH 7.2.

   Place 800 mL of UP water in a 1 L glass beaker, add 1.21 g Tris-base and stir with a magnetic agitator until dissolved, and adjust to pH 7.2 with 6 M HCl and increase the volume to 900 mL with UP water (this will be made up to 1 L with the protease inhibitor solution). Store at 4 ºC and immediately before use, add 100 µL/mL of a 10-times concentrated solution (10 X) of protease inhibitor to the volume of buffer required for extraction.
9. B2 - Myofibrillar protein extraction buffer: 10 mM Tris-HCl, pH 7.2, 0.6 M NaCl (sodium chloride), 1.7 % SDS (sodium dodecyl sulfate), 100 mM DTT. Add 600 mL of UP water to a 1 L glass beaker and add 1.21 g Tris-base and stir with a magnetic stirrer until dissolved, and adjust to pH 7.2 with 6 M HCl, then add 35.07 g NaCl and 17 g SDS and leave the solution standing at room temperature until the SDS is dissolved, only then gently mix with a magnetic stirrer taking care to avoid the formation of a foam. Make up to 800 mL in a volumetric flask by adding UP water. Store at 4 ºC. Immediately before use, add 100 µL/mL of 10 X protease inhibitor, and 100 µL/mL of 1 M DTT to the volume required for extraction.
10. B3 - Insoluble protein extraction buffer: 8 M Urea, 50 mM Tris-base pH 8, 100 mM NaCl, 10 mM EDTA (Ethylenediaminetetraacetic acid). Add 300 mL of UP water to a 1 L glass beaker and add 3.04 g of Tris-base and stir with a magnetic stirrer until dissolved and adjust to pH 8 with 6 M HCl. To the Tris-base solution gradually add 240.24 g of Urea, 2.922 g of NaCl, 14.62 g of EDTA and mix until fully dissolved (this may take 1 – 2 hours). Make-up the final

volume of the solution to 500 mL in a volumetric flask by adding UP water and store at room temperature.

11. Tissue homogenizer: "Tissue lyser II".

2.1.2 Protein quantification

1. Bradford protein assay kit and Bovine Serum Albumin Standard.
2. 96-well flat bottomed multiwell plates.
3. Microplate reader

2.1.3 1D SDS-PAGE

1. 40 % polyacrylamide/ bis solution (37.5:1) (*see* **Note 2**).
2. Resolving gel buffer: 1.5 M Tris-HCl, pH 8.8. Dissolve 36.34 g of Tris-base in 200 mL of UP water and adjust to pH 8.8 with 6 M HCl and store at 4 ºC.
3. Stacking gel buffer: 1 M Tris-HCl pH 6.8. Dissolve 24.23 g of Tris-base in 200 mL of UP water and adjust to pH 6.8 with 6 M HCl and store at 4 ºC.
4. Glass plates (8 x 10 cm), combs (15 wells), spacers (1.5 mm) and assembly chamber.
5. Mini-PROTEAN® Tetra Cell or equivalent system for gel casting and electrophoresis.
6. 10 % (m/v) SDS in UP water.
7. Ammonium persulfate (APS): 10 % (m/v) solution in UP water (*see* **Note 3**).
8. TEMED (N,N,N′,N′-Tetramethyl ethylenediamine).
9. Whatman paper.
10. SDS loading buffer, five times concentrated (5 x): 250 mM Tris-HCl, pH 6.8, 10 % SDS (m/v), 50 % glycerol (v/v), 0.25 % bromophenol blue (w/v), 500 mM DTT.

142 In a 15 mL falcon tube mix 2.5 mL of stacking gel buffer (1 M Tris-HCl, pH
143 6.8), 1 g SDS, 5 mL glycerol, 0.025 g bromophenol blue and 0.77 g of DTT.
144 Leave to stand for 10 minutes and then vortex to mix all reagents and adjust the
145 volume to 10 mL with UP water. Prepare 10 aliquots of 1 mL and store 9 at −20
146 °C and leave one aliquot at 4 °C for use.
147 11. SDS-PAGE running buffer: 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1 %
148 SDS. Add 800 mL of UP water to a 1 L glass beaker and add 3.03 g of Tris-HCl
149 and 14.4 g of glycine and stir with a magnetic stirrer until dissolved. Add 1 g of
150 SDS to the surface of the solution and leave for 5 minutes (min) to dissolve and
151 then make up to 1 L with UP water and store at 4 ºC.
152 12. Pre-stained Protein Ladder (range 10-200 kilodaltons, KDa).
153 13. Power supply: Power Pack Universal Power Supply.
154 14. Coomassie blue (CBB) staining solution: 40 % methanol, 10 % acetic acid, 0.1
155 % Coomassie brilliant blue R-250, ddH2O (double distilled water).
156 15. Destaining solution: 40 % methanol, 10 % acetic acid, ddH2O.

157

158 **2.2 Western blotting to detect β-parvalbumin in the white muscle sarcoplasmic**
159 **fraction**

160 1. Primary antibody: monoclonal anti-parvalbumin clone PARV-19, mouse
161 ascites fluid. Store in 5 µL aliquots at -20 ºC.
162 2. Secondary antibody: anti-mouse IgG (whole molecule) – peroxidase-labeled
163 antibody, produced in rabbit. Store in 5 µL aliquots at -20 ºC.
164 3. Nitrocellulose membrane 0.2 µm.
165 4. TBS (tris-buffered saline): 20 mM Tris-base, 150 mM NaCl, deionised water
166 and adjust to pH 7.6 with 6 M HCl.

5. TBS-T: TBS, 1 % Tween 20 (*see* **Note 4**).
6. Blocking buffer: TBS-T, 5 % skimmed milk powder (w/v) (*see* **Note 5**).
7. Transfer buffer: 25 mM Tris base, 192 mM glycine, 10 % methanol, 0.01 %. SDS, ddH2O water.
8. Ponceau S staining: 1.3 mM (0.1 %) Ponceau S tetrasodium salt, 0.874 M acetic acid, deionised water.
9. Whatman paper.
10. Roller, pencil, scissors, smooth-ended tweezers, ruler.
11. Plastic or glass container for "transfer unit*"* preparation and small plastic container for incubations (eg. square petri dishes).
12. Western blotting chemiluminescent detection reagent.
13. Western blotting transfer tank (wet transfer) system with plastic cassettes and sponges.
14. Power supply: power pack, universal power supply.
15. CCD (charge-coupled device) camera that detects chemiluminescence.

3. Methods

3.1 Global protein extraction of fish white muscle for proteomic analysis

Sea bass white muscle (1 cm^3) was sampled with a clean scalpel, just below the dorsal fin and approximately mid-point of the standard length. Skin was dissected off, and the muscle wrapped in tin foil and immediately immersed in liquid nitrogen and stored at -80 ºC until protein extraction. For the protein extraction protocol all steps should be carried out at 4 ºC unless otherwise indicated. A schematic workflow of the procedure is presented in Fig. 1.

3.1.1 Protein Extraction

1. Add approximately 340 mg of silica beads (0.5 mm) with a micro spoon spatula to a 2 mL micro tube with cap and note the weight (*see* **Note 6**).
2. With a sterile scalpel quickly cut-off approximately 350 mg of white muscle, register the weight, wrap in aluminum foil, dip in liquid nitrogen and smash with a hammer.
3. Place the muscle fragments in a cold mortar, add liquid nitrogen and create a fine power using the pestle (keep frozen throughout by adding liquid nitrogen as necessary) (*see* **Note 7**).
4. Add approximately 125 mg of powdered muscle to the tube containing the silica beads, weigh and note the weight of added tissue. Hold the tubes at -80 ºC until sample extraction.
5. Add the sarcoplasmic protein extraction buffer (B1); for 0.5 g of tissue add 2 mL buffer.
6. To extract the sarcoplasmic proteins (FSc) insert the sample tubes into a tissue homogenizer and program 2 cycles of 20 seconds (s) with an interval of 30 s between each cycle (*see* **Note 8**).
7. Centrifuge at 28,000 x *g* for 15 min at 4 ºC.
8. Collect the FSc supernatant into a new tube. Take an 80 µL aliquot of FSc and store at -20 ºC for immediate use and store the remaining FSc extract at -80 ºC.
9. Solubilize the pellet containing the myofibrillar protein (FM) fraction with extraction buffer (B2) (using 2 mL for 0.5 g pellet). Resuspend the FM fraction by gently pipetting up and down and vortexing for 1 min.

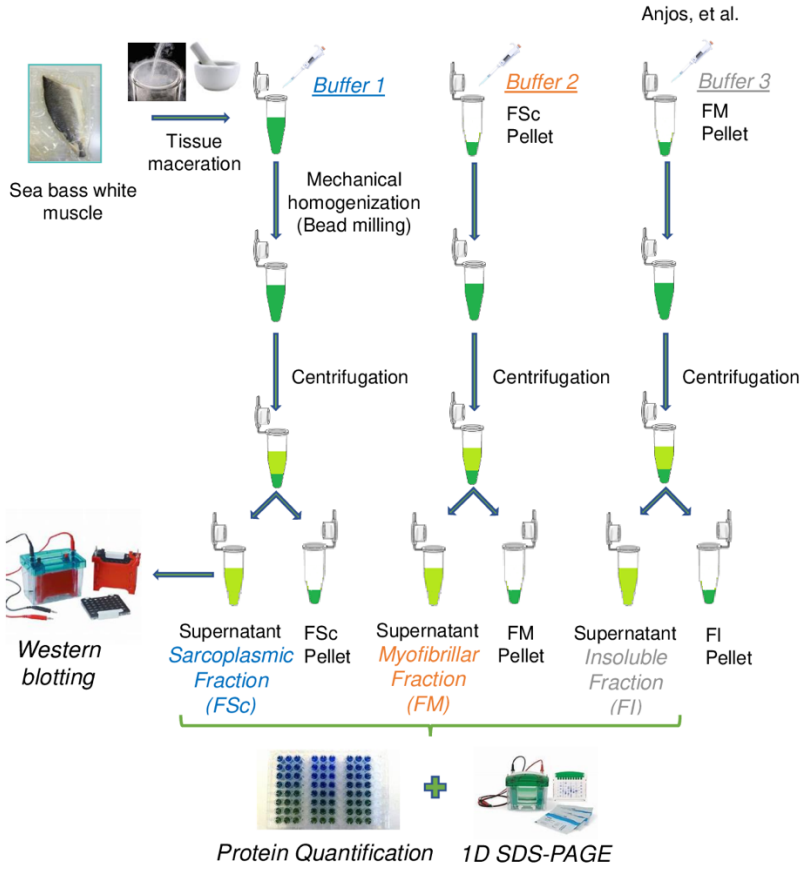


Fig. 1 Overview of the general experimental workflow used for global protein extraction of white muscle from fish for proteomic analysis and detection of allergens by Western blotting. The white muscle used for extraction in the study was from the European sea bass (Dicentrarchus labrax). Following tissue maceration (combining liquid nitrogen and mechanical homogenization by bead milling), the sarcoplasmic (FSc—Blue), myofibrillar (FM—orange), and insoluble (FI—gray) protein fractions were sequentially extracted using appropriate buffers (Buffer 1, 2, and 3). The extraction buffers were chosen taking into consideration the biochemical characteristics of each of the muscle protein fractions. The protein concentration of the different fractions was quantified using the Bradford method and the protein extracts were analyzed by 1D SDS-PAGE (12%). The sarcoplasmic fraction (FSc) was also analyzed by Western blotting to identify β-parvalbumins.

10. Heat the FM protein extract at 95 ºC for 5 min, vortex briefly and then heat at 95 ºC for 5 min. Allow to cool to room temperature.
11. Centrifuge at 28,000 x *g* at 22 ºC for 15 min.
12. Collect the FM protein supernatant into a new tube. Take an 80 µL aliquot of FM and store at -20 ºC for immediate use and store the remaining FM extract at -80 ºC.

13. Solubilize the insoluble muscle protein in the pellet with extraction buffer B3 (using 1 mL for 0.5 g pellet). Resuspend the insoluble muscle protein pellet by gently pipetting up and down and vortexing for 1 min.
14. Centrifuge at 28,000 x *g* for 15 min at 20 ºC.
15. Collect the insoluble muscle protein supernatant (FI) into a new tube. Take a 50 µL aliquot of FI and store at -20 ºC for immediate use and store the remaining FI extract at -80 ºC.

3.1.2 Protein Quantification

To proceed with subsequent protein quantification, a colorimetric assay based on the Bradford method can be used [20]. Several kits are available and the Quick Start™ Bradford protein assay kit is appropriate for measuring the FSc, FM and FI protein extracts prepared from sea bass white muscle. To save time and reagents a 96-well microplate assay can be used, and a standard curve prepared using bovine serum albumin (BSA) with a linear range of 0.125–2 mg/mL is included in each assay. Follow the protocol provided with the kit used and remember to include appropriate controls for each of the protein extraction protocols (*see* **Note 9**).

3.1.3 Proteome profile analysis by 1D SDS-PAGE

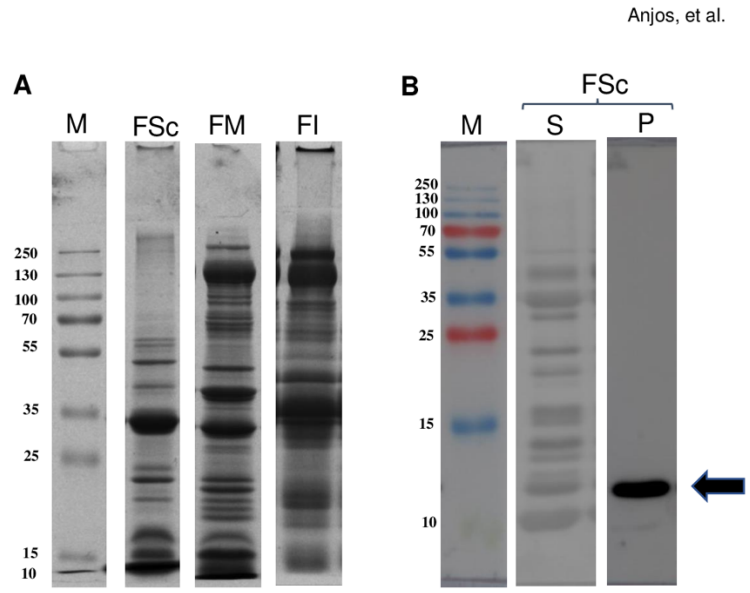
One-dimensional (1D) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is used to assess the quality of the protein extracts and the proteome profile of sea bass white muscle. The protocol described below uses a vertical gel electrophoresis system, with a standard mini gel set-up (8 x 10 cm) of 1.5 mm of thickness and a comb with 15 wells.

1. Warm the reagents to room temperature and ensure the glass plates, combs, and spacers are clean and dry. Prepare the glass gel cassettes according to the instructions of the manufacturer.
2. Prepare the resolving gel (12%) by mixing 3 mL of polyacrylamide, and 4.296 mL water in a 50 mL beaker with a magnetic stirrer. Degas with a water pump for 10 min and then add 100 µL of SDS, 100 µL of APS, and 4 µL of TEMED and rapidly cast the gels and overlay with isobutanol or water (*see* **Note 10**).
3. Prepare the stacking gel (5%) by mixing 0.630 mL of resolving buffer, 0.625 mL of polyacrylamide, 3.640 mL water, 50 µL of SDS, 50 µL of APS, and 5 µL of TEMED and cast on top of the polymerized resolving gel from which the isobutanol or water has been removed. Immediately after casting the stacking gel insert the comb without introducing air bubbles and only remove it when the gel has polymerized.
4. For sample preparation mix 15 µg of total protein from FSc, FM and FI samples (*see* Subheading **3.1.2**) with 2 µl of 5 x SDS loading buffer. To facilitate loading make up all samples to the same final volume (e.g. 10 µL) using the appropriate extraction buffer for samples of each of the muscle fractions.
5. Denature the samples by boiling at 95 ºC for 5 min in a dry bath, centrifuge at 14,000 g at room temperature for 3 min and leave at room temperature until loaded onto the polyacrylamide gel (*see* **Note 11**). Do not add lysis buffer to the prestained protein ladder or subject it to heat.

6. Place the polyacrylamide gels into an electrophoresis chamber (*see* **Note 12**), fill the upper and lower buffer chamber with the running buffer and wash-out the sample wells of the stacking gel with running buffer.
7. Load the protein samples into the wells of the stacking gel (one sample/well); add to one of the wells a protein ladder for sizing the proteins after electrophoresis.
8. Run the polyacrylamide gel at a constant current (30 mA) until the dye front reaches the bottom (takes around 90-100 min). Run the electrophoresis at 4 ºC to avoid overheating, since this reduces electrophoresis resolution.
9. After electrophoresis, remove the polyacrylamide gels and release them from the glass-cassette with a flat spatula. Rinse the gels in water for approximately 5 min and then stain them with Coomassie blue (CBB) (50 mL/gel) by shaking for 1 h at room temperature.
10. Substitute the CBB with the destain solution and incubate the polyacrylamide gels in several changes of destain until blue staining protein bands are clearly visible. Document with an imager. A typical protein profile of different fractions of fish white muscle extracts is presented in Fig. 2A.

3.2 Western Blotting to Detect *β*-Parvalbumin in the Fish White Muscle Sarcoplasmic Fraction

Prior to Western blotting, a titration assay can be performed to optimize the working dilutions of primary and secondary antibodies using the Dot Blot technique (not described here). Plan your experimental work in advance (define samples and antibody dilutions to be tested, membrane type and working buffers) (*see* **Note 13**). It may be difficult to obtain commercial antibodies that specifically detect allergenic proteins in fish as few are available in the market.



304

305 **Fig. 2** (**a**) Coomassie blue stained 1D SDS-PAGE (12% polyacrylamide) of the proteins extracted from
306 the sarcoplasmic fraction (FSc), myofibrillar fraction (FM), and insoluble fraction (FI) of the European
307 sea bass white muscle. (**b**) Western blot of β-parvalbumin (P) in soluble sarcoplasmic protein extracts of
308 European sea bass white muscle. Proteins in FSc were transferred to the membrane after electrophoresis
309 by 1D SDS-PAGE (15% polyacrylamide). The FSc total protein profile was identified by Ponceau S
310 staining (S). M molecular weight marker (in kDa). Note the strong immunoreaction highlighted by a
311 black arrow

312

313 1. Prepare and run the samples (FSc extracts and the pre-stained protein ladder) on
314 a 1D SDS-PAGE gel (*see* Subsection **3.1.3**, 1 – 8).
315 2. On completion of electrophoresis rinse the gel (still supported by one of the
316 glass plate) in UP water to remove traces of SDS-PAGE running buffer.
317 3. Trim the gel to the appropriate size, removing the stacking gel and any excess
318 gel without protein and incubate it in 50 mL of transfer buffer for at least 10
319 min.
320 4. Accurately measure the dimensions of the gel and prepare a piece of
321 nitrocellulose membrane and 8 pieces of Whatman paper (Nº 1) with the same
322 dimensions (*see* **Note 14**).

5. Equilibrate the nitrocellulose membrane with the transfer buffer by soaking it for 5 min in 50 mL of distilled water followed by 10 min in transfer buffer (*see* **Note 15**).
6. Soak the transfer cassette, sponges and Whatman paper (Nº 1) in transfer buffer. Build the "*sandwich*" by placing the gel on the 4-sheet stack of Whatman paper, then overlay the gel with the nitrocellulose membrane and then the remaining 4 sheets of Whatman paper. Gently pass a roller over the "stack" to remove air bubbles that will impede protein transfer.
7. Place your "*sandwich*" in the cassette between the two wet sponges and lock the cassette (*see* **Note 16**).
8. Fill your transfer tank with transfer buffer at 4 ºC, add a magnetic stirrer to agitate the buffer during transfer and then introduce the cassette (*see* **Note 17**).
9. Put the lid on the apparatus (pay attention to the orientation of the electrodes to ensure that proteins will transfer to the nitrocellulose membrane) and initiate transfer at 4 ºC under a constant current (300 mA) for 1 h (*see* **Note 18, 19**).
10. After protein transfer from the polyacrylamide gel to the nitrocellulose membrane remove the gel and stain it in Coomassie blue (*see* Subsection **3.1.3**) to confirm that protein transfer occurred.
11. Cut a very small portion off the right-hand corner of the membrane (to facilitate identification of membrane orientation in subsequent steps). Then wash the membrane for 5 min in UP water at room temperature (RT) with gentle agitation (90 rpm) (*see* **Note 20**). From this step onwards, all the membrane incubation steps can be done at room temperature unless indicated otherwise.
12. To confirm protein transfer occurred to the nitrocellulose membrane incubate it in 50 mL of Ponceau S for 5 min and wash it quickly in deionised water until

the protein bands became visible. Take a picture of the nitrocellulose membrane and eliminate the Ponceau S staining by washing in deionised water for 5 min or until the red bands disappear**.**

13. Incubate the nitrocellulose membrane in TBS-T for 5 min and then place it in blocking buffer at 4 ºC overnight or for 1 h at room temperature (*see* **Note 21, 22**).
14. Incubate the nitrocellulose membrane with the primary antisera at an appropriate dilution (*β*-parvalbumin, 1:5000 in TBS) and incubate for 1h at room temperature and then remove excess antisera by washing the membrane twice in TBS-T for 5 min, followed by 5 min in TBS (*see* **Note 23, 24**).
15. Incubate the nitrocellulose membrane with the secondary antibody conjugated to horse radish peroxidase (1:80,000 in TBS) for 1 h at room temperature and then wash twice in TBS for 5 min.
16. Develop with Western blotting chemiluminescent detection reagent following the manufacturer´s instructions (*see* **Note 24**) and detect the chemiluminescence with a CCD camera (exposure time is adjusted as required, in the case of *β*-parvalbumin, with the antisera dilutions used 3 min exposure worked well). Fig. 2B presents the results of Western blotting for *β*-parvalbumin in the sea bass white muscle FSc fraction.

4. Notes

1. 9 L of liquid nitrogen is sufficient for approximately 60 samples.
2. Respect the safety rules and consult the safety data sheets (MSDS) of each reagent prior to use, e.g., acrylamide as a monomer is considered toxic, directly affecting the nervous system, and it may be a carcinogen. Acrylamide is readily

absorbed through intact skin from aqueous solutions. Wear gloves and a laboratory coat when preparing the acrylamide gel solution.

3. The APS should be made fresh.
4. Tween 20 is viscous; it may be easier to use a measuring cylinder to measure the necessary volume instead of a pipette.
5. Ensure the milk powder is well dissolved by mixing it with a magnetic stirrer for 10 min. You can store the blocking buffer at -20 ºC and reuse it several times.
6. Approximately 340 mg of silica beads (0.5 mm) were weighed by adding 3 spoons of silica beads.
7. Use safety glasses and gloves.
8. Take a photograph of one tube.
9. To determine the protein concentration: calculate the average of the readings obtained for the blank and subtract this value from the readings obtained for the standards and samples. Plot the standard curve by plotting the 595 nm values (y-axis) versus their concentration in mg/mL (x-axis). Determine the unknown sample concentration using the absorbance and reading off the corresponding concentration from the standard curve. If the samples were diluted before protein determination, adjust the final concentration of the unknown samples by multiplying by the dilution factor (e.g. 1/10).
10. The APS should be made fresh immediately before use and the TEMED should be added immediately before pouring the gel (both reagents are initiators of the acrylamide polymerization). Agitate the solution slowly while adding the reagents to avoid bubble formation. Water, isobutanol or 80 % isopropanol in ddH_2O can be used to cover acrylamide gels undergoing polymerization, to

397 prevent contact with atmospheric oxygen (which inhibits acrylamide
398 polymerization).
399 11. Centrifuging the samples prior to the SDS-PAGE helps remove insoluble
400 debris, which can lead to streaking in the protein lanes (evident when stained
401 with Coomassie blue).
402 12. Always follow the instructions of the manufacturer.
403 13. Always consider the recommendations of the producer of the antibodies as well
404 as the nitrocellulose membranes (or suitable alternatives) and if possible, take
405 into account the characteristics of the antigens (eg. irrespective of protein
406 structure the antibody recognizes the antigen epitope).
407 14. Use a carbon pencil and a ruler and do not touch the membrane with bare hands.
408 15. In our experiment we used a nitrocellulose membrane, but (Polyvinylidene
409 difluoride) PVDF membranes can also be used. If a PVDF membrane is used
410 soak it for 10 sec in 100 % methanol, followed by distilled water for 5 min
411 followed by TBS for 10 min.
412 16. You can leave it the whole time, always carry out the transfer at 4 ºC.
413 17. Check that there are no bubbles inside the tank to ensure a steady and
414 homogeneous flow of electricity through the polyacrylamide gel.
415 18. Always introduce the membrane on the positive facing side of the transfer
416 cassette, as the proteins in the polyacrylamide gel are negatively charged and
417 migrate towards the positive pole.
418 19. This current intensity is effective for the transfer of parvalbumin, the transfer
419 current can be calculated as 2 mA /h/ cm^2. The time of transfer should be
420 optimized for the proteins under study.

20. This wash step is very important to remove residual SDS, which is an inhibitor of Ponceau S staining.
21. In our experiments the working buffer used was TBS, but you can also use phosphate buffered saline (PBS) or phosphate buffer (PB), unless alkaline phosphatase is used for signal detection.
22. The volume of the washing buffer and blocking buffer can be calculated as approximately 2 mL / cm^2 of nitrocellulose membrane.
23. Primary antibody dilutions should be prepared in sterile TBS and under sterile handling conditions so that these working solutions can be reused and to minimize microbial contamination.
24. Development with ECL plus start kit according to the instructions, remember a) remove the kit from the fridge 20 min before development and b) remove excess TBS buffer by blotting with Whatman paper.

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530 **Figure Captions**

531 **Figure 1**. Overview of the general experimental workflow used for global protein
532 extraction of white muscle of fish for proteomic analysis and detection of allergens by
533 Western blotting. The white muscle used for extraction in the study was from the
534 European sea bass (*Dicentrarchus labrax*). Following tissue maceration (combining
535 liquid nitrogen and mechanical homogenization by bead milling), the sarcoplasmic
536 (FSc-Blue), myofibrillar (FM-orange) and insoluble (FI-grey) protein fractions were
537 sequentially extracted using appropriate buffers (Buffer 1, 2, 3). The extraction buffers
538 were chosen taking into consideration the biochemical characteristics of each of the
539 muscle protein fractions. The protein concentration of the different fractions was
540 quantified using the Bradford method and the protein extracts were analyzed by 1D
541 SDS-PAGE (12%). The sarcoplasmic fraction (FSc) was also analyzed by Western
542 blotting to identify *β*-parvalbumins.

543

544 **Figure 2**. (**A**) Coomassie blue stained 1D SDS-PAGE (12 % polyacrylamide) of the
545 proteins extracted in the sarcoplasmic fraction (FSc), myofibrillar fraction (FM) and
546 insoluble fraction (FI) of the European sea bass white muscle. (**B**) Western blot of *β*-
547 parvalbumin (P) in soluble sarcoplasmic protein extracts of European sea bass white
548 muscle. Proteins in FSc were transferred to the membrane after electrophoresis by 1D
549 SDS-PAGE (15 % polyacrylamide). The FSc total protein profile was identified by
550 Ponceau S staining (S). M: Molecular weight marker (kDa). Note the strong
551 immunoreaction highlighted by a black arrow.

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