Chapter 12

Analysis of Proteins from Marine Molluscs

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Summary

Application of the two-dimensional gel electrophoresis (2DE) protocols which were developed for samples of mammalian origin gives unsatisfactory results when used in samples from marine molluscs. This chapter describes a detailed protocol of 2DE that can be applied to these organisms, especially for *Ruditapes decussatus* and *Bathymodiolus azoricus*.

Key words: Two-dimensional electrophoresis (2DE), marine bivalves, *Ruditapes decussatus*, *Bathymodiolus azoricus*.

1. Introduction

Marine molluscs, particularly mussels, oysters, and clams, have been used worldwide as bioindicators to assess the impact of pollutants in coastal marine ecosystems and their health status. More recently, the discovery in 1997 of large mussel beds in deep sea hydrothermal vents *(1)*, particularly in the Mid-Atlantic Ridge, has attracted much scientific attention due to their capacity to live in one of the most extreme environments on earth characterized by high temperature and pressure, low pH, enriched in toxic sulphide species *(2)*, radionuclides, and naturally high bioavailable metal concentrations that would be toxic or even lethal to coastal marine species *(3, 4)*. Therefore these species have been considered as models to assess pollution effects in natural contaminated environments *(5)*.

The isolation of proteins from marine bivalves from coastal or extreme environments, like deep sea hydrothermal vents, using two-dimensional electrophoresis (2DE) was proved not to be straightforward when using standard protocols developed for

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mammalian tissues mainly due to the excess of salt water and the lipid content of some of particular tissues studied. Recently 2DE protocols were developed for bivalve species in particularly the coastal clam *Ruditapes decussatus* and the deep sea hydrothermal vent mussel *Bathymodiolus azoricus*. Two tissues were used: gills and digestive gland. 2DE gels (18 × 18 cm) were able to separate more than 2,000 proteins.

Although the genome of these species is unknown, a number of proteins have already been sequenced. Some relevant proteins were separated from the spots and some of them sequenced. In bivalves, gill and digestive gland tissues usually do not provide good resolution 2DE gels because they may contain exogenous proteins. To overcome this problem in this protocol we describe the best technique for protein separation using 2DE.

2. Materials

2.1. Equipment

IKA, model Ultra-Turrax TD 25, Ettan IPGphor II (GE Healthcare), Protean II xi cell (Bio-Rad, Labs). Vertical system.

2.2. Solutions and Reagents

1. *Homogenization buffer*: 10 mM HEPES and 250 mM Sucrose, 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM PMSF, 10% protease inhibitors (protease inhibitor tablets, Sigma, Aldrich). DTT, PMSF, and protease inhibitors are added just prior to use. This can be stored at 4°C for 1 week.
2. *Precipitation solution*: 10% trichloroacetic acid (TCA) in acetone containing 20 mM DTT. DTT is added just prior to use. This can be stored at 4°C for 1 week.
3. *Sample dilution buffer*: 7 M urea (GE Healthcare), 2 M thiourea (GE Healthcare), amberlite MB-150 1% (Sigma, Aldrich) *(6)*. Filter solution through 0.20-μm filter. Add 4% CHAPS (GE Healthcare), 0.8% Pharmalyte (GE Healthcare), 65 mM DTT (GE Healthcare), 10% isopropanol *(6)*, and a few grains of bromophenol blue (BPB). Store in 2.5 mL aliquots at –70°C for 2 months.
4. *SDS equilibration buffer*: urea 6 M, Tris–HCl, pH 8.8, 75 mM, glycerol 3%, SDS 4%, and a few grains of bromophenol blue (BPB). Store aliquoted. Can be stored at –20°C for 2 months (*see* **Note 1**).
5. *Electrophoresis buffer*: SDS: 0.1%, Tris base: 25 mM, Glycine: 192 mM. Store at room temperature.
6. *10% SDS solution*: Filter solution through a 0.45-μm filter and store at room temperature.

7. *10% ammonium persulphate solution*: Prepare just prior to use.
8. *Agarose sealing solution*: 0.5% agarose. Add all ingredients into a 500 Erlenmeyer flask. Swirl to disperse. Heat on a heating stirrer until the agarose is completely dissolved (*see* **Note 2**). Dispense 2 mL aliquots and store at room temperature.
9. *EDTA 500 mM*: store at room temperature.
10. Immobiline DryStrip, pH 3–10 NL, 18 cm (GE Healthcare), immobilized pH-gradient isoelectric focusing gels for the first-dimension separation step.

2.3. Software

PdQuest (Bio-Rad, Laboratories, Hercules, CA).

3. Methods

3.1. Preparation of Animals

In contamination experiments animals should be acclimated in clean sea water for 7 days prior to the beginning of the experiment *(7)* (*see* **Note 3**).

3.2. Protein Sample Preparation

1. Dissect tissues at 4°C, using a scalpel cooled by dipping periodically into liquid nitrogen. It is convenient to use a pair of thin cotton gloves during these procedures to avoid transferring heat to the samples.
2. Collect tissues in 5-mL screw-capped plastic tubes on liquid nitrogen.
3. When exposed to the cold, the tissues immediately freeze and can subsequently be stored at –70°C or below (*see* **Note 4**).

3.3. Total Protein Extraction

Having obtained clean samples, it is very important to mince them effectively. Failure to do so will result in selective extraction of protein, which will distort the results of the experiment (*see* **Note 5**).

1. Suspend each sample in three volumes of homogenization buffer and homogenize in 15-mL tubes, using an Ultra-Turrax IKA-Werke homogenizer at 4°C in a cold room, on ice (*see* **Note 6** and Chapter "Difficult Proteins").
2. Centrifuge the homogenate at 15,000 × *g* for 2 h at 4°C.
3. Transfer the supernatant into 2.5-mL Eppendorf tubes for subsequent quantification of sample protein (*see* **Subheading 3.6**).
4. Prepare aliquots of sample so that the final quantity of protein is 300 µg.

3.4. Precipitation Procedure

Protein precipitation is an optional step in sample preparation for 2DE. Precipitation is generally employed selectively to separate proteins in the sample from contaminating species such as salts, detergents, nucleic acids, lipids, etc. that might otherwise interfere with the 2DE result *(8)*.

1. Suspend each sample in nine volumes of precipitation solution (*see* **Note 7**).
2. Precipitate proteins for at least 2 h at –20°C.
3. Pellet proteins by centrifugation at 10,000 × *g* for 30 min.
4. Wash pellet with cold acetone (*see* **Note 8**).
5. Remove residual acetone by air drying.

3.5. Protein Solubilization

1. Resuspend the precipitate in the sample dilution buffer. Use 300 µg in 300 µL, vortex, and leave for 30 min.
2. Centrifuge at 14,000 × *g* for 10 min (*see* **Note 9**).

3.6. Quantification of Sample Protein

Quantification of the proteins concentration was carried out using ovalbumin as a protein standard using the Bradford method *(9)*.

3.7. Isoelectric Focusing

Isoelectric focusing is an electrophoretic method that separates proteins according to their isoelectric points (pI). For this separation wide-range immobilized pH-gradient (IPG) gels, with pH values ranging from 3 to 10, in 18-cm length strips, were used (*see* **Note 10**).

About 300 µg of total extractable protein can be resolved by IEF on immobiline ® DryStrip, pH 3–10, NL, 18 cm (Pharmacia Biotech).

1. Pipette the samples (prepared as described in the **Subheading3.5**) into each strip holder. Distribute the solution evenly over the channel length and remove any large bubbles.
2. Carefully remove the cover foil from the Immobiline DryStrip, starting from the anodic end (+ end).
3. Carefully place the Immobiline DryStrip into the holder channel, gel-side down (*see* **Note 11**).
4. Overlay the strip with Immobiline DryStrip Cover Fluid to minimize evaporation and prevent urea crystallization.
5. Apply the pressure blocks on the underside of the cover to ensure that the Immobiline DryStrip gel maintains good contact with the electrodes as the gel swells.
6. Ensure that the strip holders are properly positioned on the Ettan IPGphor II platform. Use the guide marks along the sides of the platform to position each strip holder and check that the pointed end is over the anode (pointing to the back of the unit) and the blunt end is over the cathode. (Please refer

to the Ettan IPGphor II user manual for complete details). Check that both external electrode contacts on the underside of each Strip Holder make metal-to-metal contact with the platform.

7. Close the Ettan IPGphor II lid. Program the first step of Ettan IPGphor II to make an active rehydration during 12 h under low voltage (20 V) at low current (50 μA/IPG strip), at 20°C (*see* **Note 12**).
8. Program the other steps of the IEF: 1,000 V, 1 h; 4,000 V, 8,000 V, 1 h, and 8,000 V, until 50,000 V (~5 h) (*see* **Note 13**).
9. After IEF is complete, proceed to the second-dimension separation immediately or store the Immobiline DryStrip gels at −60°C or below in screw-capped tubes.

3.8. Second-Dimension SDS–PAGE

In the second-dimension separation, where proteins are separated by molecular weight, traditional SDS–PAGE is employed.

3.8.1. Equilibrating Immobiline DryStrip Gels

1. Place the IPG strips in individual tubes, with the support film towards the tube wall.
2. Add 5 mL of SDS equilibration buffer, 100 mg DTT per strip, 0.5 μL EDTA.
3. Cap or seal the tubes with flexible paraffin film and place them on their sides on a rocker for the equilibration process. Equilibrate for 15 min.
4. Pour off buffer from earlier step and add the appropriate volume of SDS equilibration buffer, 250 mg iodoacetamide, 0.5 μL EDTA to each strip. Again cap or seal the tubes with flexible paraffin film and place them on their sides on a rocker for the equilibration process. Equilibrate for an additional 15 min (*see* **Note 14**).

3.8.2. Electrophoresis Using a Vertical Electrophoresis System

Cast the 10% polyacrylamide gels using 30% Acrylamide/Bis solution (Bio-Rad Laboratories, Hercules, CA) in 1-mm cassettes (*see* **Note 15**).

1. Place the strip with the acidic end to the left, gel surface up onto the protruding edge of the longer glass plate.
2. With a thin plastic ruler, gently push the Immobiline dry strip gel down so that the entire lower edge of the Immobiline DryStrip gel is in contact with the top surface of the gel (*see* **Notes 16** and **17**).
3. Seal the Immobiline DryStrip gel in place with the agarose sealing solution (*see* **Note 18**).
4. Insert the cassettes into the tank and pour the electrophoresis buffer to the fill line.
5. Close the lid and connect the power leads to the power supply.

6. Electrophoresis is performed at constant current (90 A) in two steps. During the initial migration and stacking period (15 min), the current is 80 V, and during the separation we apply 200 V (*see* **Note 19**).
7. Stop electrophoresis when the dye front is approximately 1 mm from the bottom of the gel (*see* **Note 20**).

4. Notes

1. This is a stock solution. Just prior to use, add DTT or iodoacetamide, for first and second equilibration, respectively, and the EDTA 0.1 mM.
2. Do not allow the solution to boil over.
3. The animals should have the same size to ensure an equal sample.
4. It is very important that the tissues never defrost between collection and solubilization in electrophoresis buffer in order to avoid proteolysis.
5. Direct grinding of frozen tissues in liquid nitrogen does not result in a sufficiently fine mincing for efficient extraction.
6. Start 30 s with a low speed and two times 15 s in a high speed. In this step the homogenate can be frozen at –70°C.
7. This approach limits proteolysis and other protein modifications. Protease inhibitors were found to be efficient enough to stop the protease activities in the sample preparation process. A TCA–acetone treatment was employed in addition to PMSF where acid and organic solvent denature almost all proteins including proteases.
8. Residual TCA must be removed by extensive washing with acetone because extended exposure to this low pH solution may cause some protein degradation or modification.
9. This step helps to precipitate remaining salts.
10. These varied pH intervals allow fine-tuning of each separation strategy to increase first-dimension loading and resolve a greater number of spots from crowded areas, and they are available with strip lengths of 7, 11, 13, 18, and 24 cm. Choose shorter strips, i.e. up to 13 cm, for fast, cost-effective screening, or when the most abundant proteins are of highest interest. The shortest IPG strips give the fastest results, but their sample load is limited. Use longer strips, i.e. 18- and 24-cm strips, for maximal resolution and loading capacity. Longer strips allow detection of more spots and make it easier

to select and identify the proteins in the map, but require longer times in both the first- and the second-dimension separations.

11. To help coat the entire gel, gently lift and lower the strip and slide it back and forth along the surface of the solution. Be careful not to trap bubbles under the Immobiline dry strip gel.
12. The active rehydration facilitates the entry of high-molecular-weight proteins into the strips.
13. Many factors affect the amount of time required for complete focusing, and each specific set of conditions, e.g. sample and rehydration solution composition, Immobiline dry strip gel length, and pH gradient. Ramping the voltage slowly while the sample is entering the IPG strip improve results.
14. Be consistent with the timing of the equilibration steps.
15. The composition of this gel should be selected to resolve proteins in the MW range of interest. Thinner gels stain and destain more quickly and generally give less background staining. Thicker gels have a higher protein capacity. Thicker gels are also less fragile and easier to handle.
16. Ensure that no air bubbles are trapped between the Immobiline drystrip gel and the slab gel surface.
17. The MW marker proteins can be applied to a paper piece, then pick up the application piece with forceps and apply to the top surface of the gel next to one end of the Immobiline DryStrip gel.
18. The agarose sealing solution prevents the Immobiline dry strip gel from moving or floating in the electrophoresis buffer.
19. For these vertical systems, cooling is optional. However, temperature control improves gel-to-gel reproducibility, especially if the ambient temperature of the laboratory fluctuates significantly. For best results, gels should be run at 25°C.
20. After this step silver staining is the most sensitive staining technique (*see* Chapter "Silver Staining of Proteins in 2DE Gels"). For protein identification we use PDQuest, Bio-Rad, Laboratories, Hercules, CA.

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