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Seagrass Omics: How to Short the Workflow for Protein Expression Analyses

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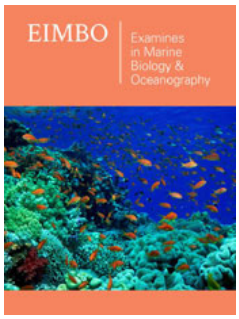

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Abstract

When planning a differential protein expression trial in seagrass populations, time consuming and expensive workflow is the main issue to be addressed. Here we reported a faster procedure from the electrophoretic separation to the mass spectrometric analysis of *Posidonia oceanica* leaf proteins, selected among three different acrylamide/diacrylamide concentrations, three different running times of denaturing electrophoresis followed by in-gel digestion reactions, three total mass spectrometry run times. We demonstrated that the procedure using 12 or 10% polyacrylamide gel, the SDS-PAGE duration of 15 min and the total LC-MS/MS run of 120 min, did not affect the proteolytic efficiency of trypsin, improved the quality of the generated peptides, increased the number of identified proteins belonging to many functional categories per sample, compared to those of a standard workflow and of the other tested conditions.

Keywords: Seagrasses; Proteomics; In-gel digestion; SDS-PAGE; Mass spectrometry

Introduction

Since the last decade, proteomic technologies have been successfully applied to marine plants to obtain insights into their adaptive capacity and gene expression plasticity against environmental threats [1-7]. In seagrasses several species-specific multistep workflows for free-label differential protein expression have been optimized to obtain high quality protein samples [8-10]; these protocols were routinely coupled with the tryptic *in-gel digestion* as this method offers the advantage to remove any further contaminants (e.g., detergents, salts) during electrophoresis. The generated peptides can be, then, readily analysed by LC-MS/MS to obtain the peptide mass fingerprinting. The main drawback of *in-gel digestion* is that a single SDS-PAGE gel lane, corresponding to a biological or technical replicate, is excised, divided in several slices and splitted in several independent digestion reactions [11] and thus in multiple mass spectrometry analyses. This is a time lengthly, costly procedure and represents the main technical issue to be faced in the differential protein expression trials. In order to improve and encourage the use of the proteomic approach to investigate at molecular level the seagrass biology and adaptation strategies, here we proposed a workflow that used a short-term electrophoresis in which protein sample was concentrated in lane of one centimeter long instead of tens; in our experience, the short-term SDS-PAGE required a single digestion reaction per sample and significantly reduced time and cost of the entire trial. Here we investigated the effects of the shortened duration of the eletrophoresis on the proteolytic efficiency, the quality of generated peptides and the number of identified proteins. Results were also discussed in terms of functional classification of the identified proteins using the Pather.db classification tools [12].

Material and Methods

Cuttings of *P. oceanica* were collected by scuba divers in a large meadows along the coast of Calabria (Souther Italy) (39,697216; 15,803951, February 2022). Leaves of each shoot have been cleaned of epiphytes, washed quickly in water, frozen in liquid N2 and stored at -80

°C. The leaf tissues were used for protein extraction as described in Piro et al. [4]. Briefly, 1gr of frozen leaves were ground in a mortar untill a fine tissue powder was obtained. The powder was divided into 2ml vials; a volume of 10% TCA in acetone was added and centrifuged at 13000rpm for 5 min at 4 °C. The pellet containing the precipitated proteins was whased in 80% acetone and dried at room temperature. For protein extraction of proteins, 0.1g of pellet was dissolved in 0.8ml of phenol (buffered withTris-HCL,pH8.0,Sigma,St. Louis, MO, USA) and 0.8ml of SDS buffer (30% sucrose, 2% SDS,0.1M Tris-HCl, pH8.0, 5% 2-mercaptoetanol) in 2ml vials. After centrifugation the phenol phase was added with five volumes of 0.1M ammoniumacetate in cold methanol, and the mixture was stored at -20 °C for 30 min to precipitate proteins. Proteins were collected by centrifugation at 13000rpm for 5 min and washed in 0,1M ammoniumacetate/methanol and 80% acetone.The final pellet containing purified proteins were dried at room temperature and dissolved at final concentration with Laemli 1D electrophoresis buffer [13]. Proteins were then quantified by the bradford assay. Protein yield was measured as mg of protein per g fresh tissue weight in three independent biological replicates for each test.

Purified protein samples were laoded on three gels of 6%, 10% and 12% polyacrylamide; short-term SDS-PAGEs were runned for 15 min, while control electrophoresis was runned for 1h and 15 min on 12% acrylamide gel at same run conditions [13]. In this way, three biological replicates for each condition have been runned; after short-term electrophoresis each replicate resulted in one large band and it has been processed as a single *in-gel digestion* reaction. As control, same replicates were separated in a classical eletrophoresis and each lane was splitted in six in-gel digestion vials (Figure 1). Mass spectra were obtained by an EASY-LC 1000 coupled to a Q-Exative Classic Benchtop Quadrupole-Orbitrap hybrid Mass Spectrometer (Thermo Fisher Scientific, Germany) at three different total run times of 45 min, 60 min and 120 min. Each control vial has been analysed at 60 min run time, resulting in 360 min total time duration per replicate. Protein inference and validation were performed with Scaffold software 4.8, unsig a setting with statistical parameters of highest stringency (Proteome Software, Inc., Portland, USA). Through the X!Tandem software peptides in samples were aligned and identified against *Zostera marina* genome sequences deposited in the NCBI databases (dowloaded on November 2022).

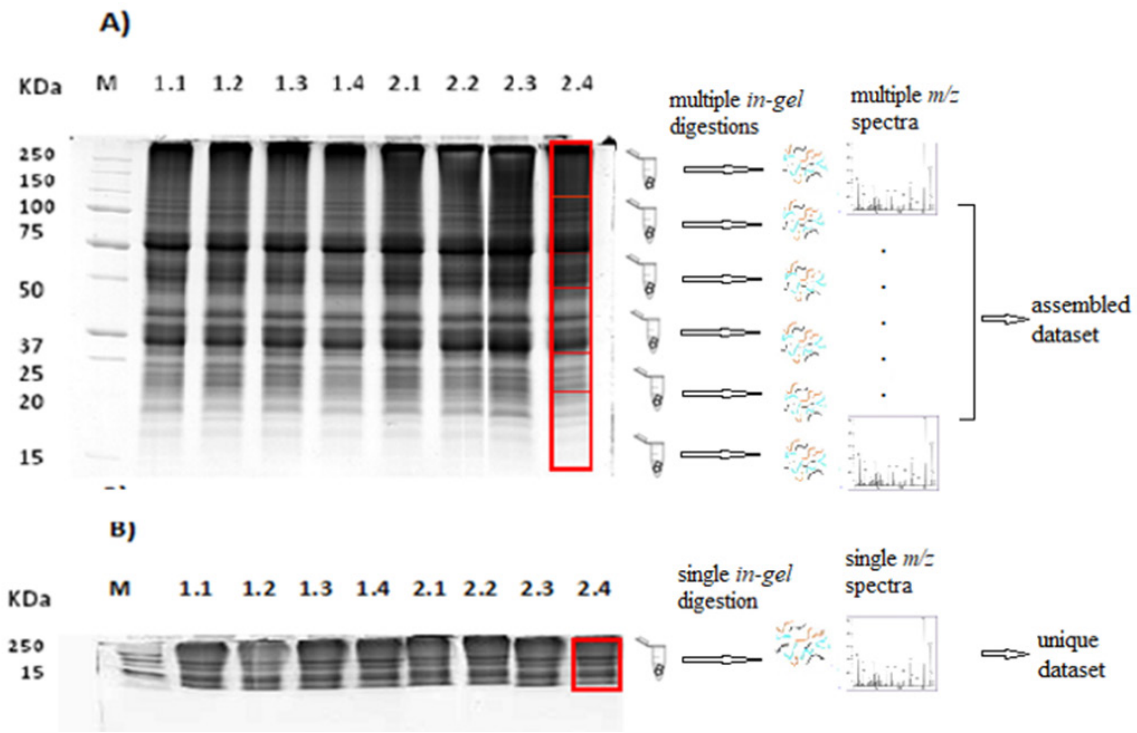

Figure 1: 12% polyacrilammide SDS-PAGEs of leaf proteins separated by A) A classic run in which lanes had a length of 10cm. Each lane was cut in six slices and processed via a typical in-gel digestion protocol followed by a multiple mass spectrometry workflow each of 60 min generating six spectra datasets that have to be assembled ; B) The shorten run in which lanes had a length of approx 1,5cm. Each lane was then cut as a single sample for processing as a single in-gel digestion, as a single mass spectrometry workflow of 120 min generating a unique spectra dataset.

Result and Discussion

Short-term SDS-PAGE Procedure using 12 or 10% acrylammide gels, 15 min run SDS-PAGE, 120 min LC-MS/MS total run time, setting at least five exclusive peptides per protein as threshold for identification significance, gave higher number of identified proteins (1977±130) respect to the 1h 15 min run replicates (1143±60), the 60 min run replicates (552±55) and 45 min run

replicates (193±25). Functional classification of proteins made by Panther.db tool [12] from the 120 min method resulted in 19 Protein classes belonging to 15 Biological processes in respect to 17 and 13 categories in control method respectively; the catergories overlapped for 70% and 86% respectively between two methods (Figure 2). Based on our results, the use of short-term gel electrophoresis improved sample processing; in fact, it did not affect tryptic digestion efficiency but generated peptides that resulted in a higher number of proteins identified than those of a classical workflow. This result suggests that within the band in short-term gel there are more proteins with very low molecular weight that, generally, could be lost during a longer electrophoretic run. The utility of short-term electrophoresis, in addition to immobilizing proteins to take advantage of gel digestion, minimizes gel length and allows to process a single digestion reaction per sample and avoid multiple runs in the mass spectrometry workflow.

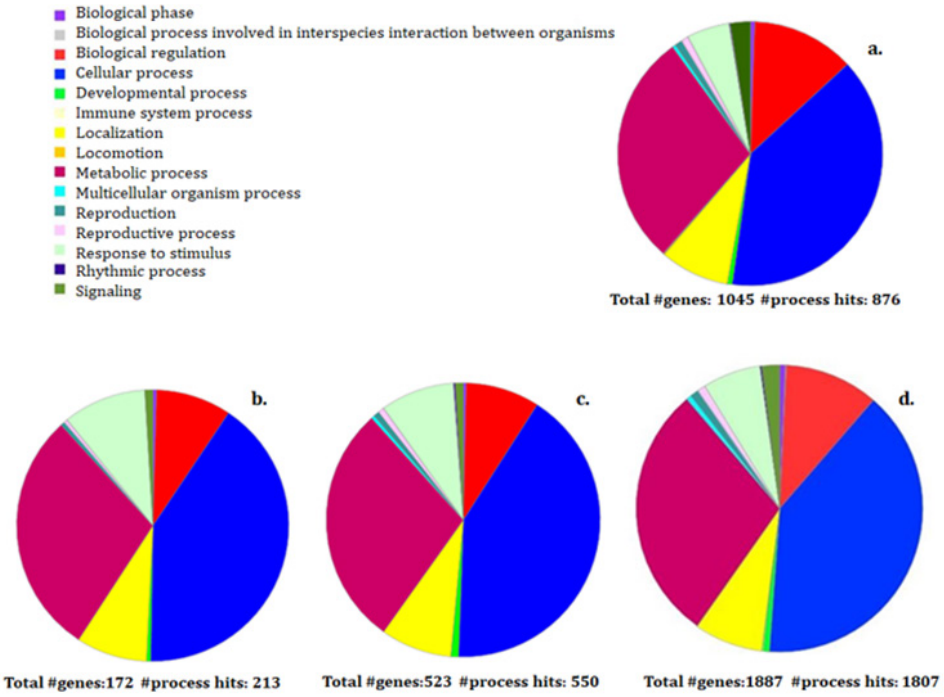

Figure 2: Functional classification by PANTHER analysis of the biological processes of the identified proteins from regular SDS-PAGE duration (a) and from short-term 12% polyacrilammide gel eletrophoresis after 45 min (b) 60 min (c) and 120 min (d) of total mass spectrometry times.

Conclusion

In our work, short-term electrophoresis coupled with a single in-gel tryptic digestion reaction reduced the time and cost required for protein separation and mass spectrometry analysis by seventy percent. The seagrass research community is strongly committed to linking ecophysiology with molecular approaches to elucidate plant responses against different environmental conditions [14-16]. Seagrass proteomics is currently able to assign significant interpretations to an increasing number of differentially expressed genes; in this context, our fine-tuned technique will allow to analyze a larger statistical sample, without having to undergo very long and expensive workflows. This result could make proteomic specialty much more feasible in differential protein expression studies of seagrass populations.

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