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Evaluation of offgel electrophoresis, electrothermal atomic absorption spectroscopy and inductively coupled plasma optical emission spectroscopy for trace metal analysis in marine plankton protein

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ABSTRACT

Two-dimensional offgel and lab-on-a-chip (LOC) electrophoresis was performed for assessing proteins from marine plankton. Proteins were isolated from plankton samples by using a phenol/sodium dodecylsulfate (SDS) buffer (2:1) pH 8.0 mixture as an extracting solution. The SDS buffer consisted of 30% (m/v) sucrose, 2% (m/v) SDS, 0.1 M of Tris–HCl, pH 8.0, 5% (v/v) β-mercaptoethanol and protease inhibitor cocktail. Before protein extraction, the plankton were subjected to different washing steps involving 10% trichloroacetic acid (TCA)/acetone and methanol. Proteins exhibiting the highest molecular weights (MWs), within the 52.6–57.3 kDa range, were found in offgel fractions of isoelectric points (pIs) ranging from 4.40 to 8.60; whereas, the highest protein concentrations were found in fraction 5 (100 μg g^{-1} for a protein of pI of 4.5 and MW of 22.0 kDa), fraction 16 (55 μg g^{-1}, protein of pI/MW of 7.4/19.1 kDa), and fraction 8 (51 μg g^{-1}, pI/MW of 5.3/22.2 kDa). Further studies were also developed for determining the amount of trace metals associated with the isolated proteins of similar pIs by electrothermal atomic absorption spectroscopy (ETAAS) and inductively coupled plasma optical emission spectroscopy (ICP-OES). Preliminary results indicate that Fe, Ni, Mn and Zn were found to be associated with proteins of variable MW (from 14 to 54 kDa) and pIs (from 3.4 to 8.2). Otherwise, Cd was found to be associated with proteins of certain pIs (6.0–6.5) and with MWs ranging from 14.1 to 53.1 kDa.

1. Introduction

In addition to the atmospheric CO_2 fixation by marine plankton in the surface ocean to produce organic carbon through the process of photosynthesis [1], marine plankton play other several roles in the marine ecosystem. Marine plankton are also considered the source of dissolved proteins in seawater. After different biochemical processes (cellular lysis, exudation) [2], beta barrel proteins and other major outer membrane proteins [3,4] from marine plankton are transferred eventually to the dissolved pool, and the survival of particular proteins in the marine environment can provide insight into the mechanisms that control the degradation of organic matter [2]. Therefore, in addition to dissolved proteins, the assessment of proteins belonging to particulate organic matters (POMs), a fraction encompassing a mixture of living POM (organisms) and non-living POM (detritus) [5], is a topic of interest.

In addition, marine plankton take up nutrients (C, N, P, and Si) and essential micronutrients (Mn, Fe, Co, Ni, Cu, and Zn) [6], and pollutants from seawater, which affect the bioavailability and transport of nutrients and pollutants in the marine ecosystem. The presence of these trace elements in the marine environment seems to have influence on the primary productivity. As an example, Mn, Fe, Co, Ni, Cu and Zn deficiencies may limit oceanic plankton production, while, an excess of some of these metals may inhibit plankton growth [7]. The importance of these micronutrients is explained taking into account the physiological functions of the different metalloproteins and/or metal-binding proteins, which can act as transporters of essential ions, or can be involved in defense mechanisms of the organism against heavy metal stress. Identification/characterization of proteins belonging to the POM fraction should therefore consider the identification of trace elements associated with them [8].

Different protein extraction procedures have been reported in the literature as a first step in proteomic studies for different materials such as plant tissues [9–11], soybean roots [12], and dinoflagellate Alexandrium sp. [11]. The protein extraction protocol depends on the sample matrix and must be optimized for each sample type to obtain the highest extraction efficiency. Regarding protein isolation from plankton, some reports can be found in the literature [13–15]. Moncheva et al. [15] have proposed the use of 20% (w/v) TCA in acetone containing 0.1% 2-mercaptoethanol and 1% SDS for protein isolation from marine phytoplankton; whereas, Jiménez et al. [14], after comparing different extraction, purification and concentration conditions, have

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proposed the treatment with 25 M Tris–HCl buffer plus a protease inhibitor as the most efficient mixture for protein extraction from a plankton based-certified reference material (BCR-414). Recently, García-Otero et al. [13,16] have also found the usefulness of a based-phenol/SDS extraction for BCR-414 proteins isolation after several TCA/acetone and methanol washing stages.

Concerning protein assessment, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as well as two-dimensional electrophoresis (2DE) have commonly been used for proteome analysis [10,11,16,17]. In general, two-dimensional gel methods are time-consuming and tedious procedures, mainly because after immobilization of pH gradient (IPG) — isoelectric focusing (IEF) when separating proteins of similar isoelectric points (pIs), several steps (gel cutting, protein extraction and cleaning up) are needed before protein separation according to their molecular weight (second dimension). Recently, the introduction of preparative IPG IEF where proteins can be recovered from liquid phase (offgel electrophoresis) allows the direct sampling of the isolated protein (first dimensional separation) into high performance liquid chromatography (HPLC) instruments as well as in microfluidic chip-based liquid chromatography [18,19]. The use of offgel fractionation techniques combined with liquid chromatography–mass spectrometry (LC–MS) and –tandem mass spectrometry (LC–MS/MS) have been reported for protein fractionation/identification mainly for clinical purposes [19–24]. On other occasions, first fractionation by offgel has been combined with conventional SDS-PAGE [11,25–27] for quantitative and qualitative sizing of proteins. Microfluidic chip-based devices such as capillary gel electrophoresis by using lab-on-a-chip technology (LOC electrophoresis) can be an alternative approach for assessing information concerning the second dimension (molecular weight sizing). This methodology has been used by several authors for qualitative and quantitative protein determinations in different matrices such as plankton, soybean cultivars, tear fluid, human granulocyte-colony, and surface and deep seawater [13,28–31]. In addition to the improved sensitivity inherent to LOC electrophoresis, low sample consumption as well as fast electrophoresis separations are other advantages of this technique [13,32–35]. The combination of offgel and LOC electrophoresis is therefore an appealing methodology which reduces the number of handling steps inherent to conventional 2DE. Since offgel electrophoresis allows recovering of proteins exhibiting similar pIs (offgel fractions) in liquid phase, a direct analysis of these fractions by conventional atomic spectrometric techniques such as ETAAS and ICP-OES can easily be addressed. Information regarding trace metals associated with proteins of similar pIs is therefore possible, which allows the selection of those trace elements associated with proteins for further protein characterization by 2DE and laser ablation (LA) — inductively coupled plasma-mass spectrometry (ICP-MS). Therefore, the aim of the current work has been the assessment of trace elements bound to the extracted proteins by simple and direct analysis of these fractions by ETAAS and ICP-OES. In addition, the optimization of offgel-LOC electrophoresis operating conditions for assessing the pIs and MWs of the isolated proteins has also been performed.

2. Materials and methods

2.1. Reagents

The pH 3–10 IPG Dry Strips, IPG Buffer 3–10 and Plus One Dry Strip cover fluid used for protein fractionation were supplied by GE Healthcare Life Science (Uppsala, Sweden). Urea (electrophoresis grade), thiourea (analytical grade) ammonium acetate and phenol solution, equilibrated with 10 mM of Tris–HCl (pH = 8.0, 1 mM ethylenediaminetetraacetic acid, EDTA) were from Sigma Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT) (electrophoresis grade), methanol, Tris–HCl and glycerol solution were purchased from Merck (Darmstadt, Germany). TCA and acetone were from Panreac (Barcelona, Spain), and β-mercaptoethanol was from Fluka (Vancouver, Canada). SDS and sucrose were purchased from AppliChem (Darmstadt, Germany). The Agilent protein 80 Kit containing the protein chips, Protein 80 Gel-Matrix, Protein 80 Dye Concentrate, Protein 80 Sample Buffer and Protein 80 Ladder was supplied by Agilent Technologies (Waldbronn, Germany). Total protein assessment was performed with a Bradford protein assay containing Coomassie Brilliant Blue G-250 and bovine serum albumin (BSA) from Thermo Scientific (Rockford, IL, USA). Isolated proteins (Bradford assay) were re-dissolved in 2-DE rehydration solution prepared with 5 M of urea, 2 M of thiourea, 2 mM of tributyl-phosphine (Sigma-Aldrich), 65 mM of DTT, 65 mM of 3-[(3-cholamidopropyl)-dimethylammonio] propanesulfonate (CHAPS) from Sigma-Aldrich, 0.15 M of N-phenyl-methyl-N,N-dimethylammonium-propane-sulfonate (NDSB-256), 1 mM of sodium vanadate (Sigma-Aldrich), 0.1 mM of sodium fluoride (Sigma-Aldrich), and 1 mM of benzamidine (GE Healthcare Life Science). Element standard solutions were prepared from Cd, Co, Cr, Cu. Fe, Mn, Ni, Pb, and Zn stock standard solutions (1.000 g L^{-1}) from Scharlau (Barcelona, Spain). BSA standard (2 mg mL^{-1}) was from Thermo Scientific (Rockford, IL, USA). Ultrapure water, resistivity 18 M Ω cm (25 °C), was obtained from a Milli-Q water-purification system (Millipore).

2.2. Instruments

Two-dimensional electrophoresis was performed with a 3100 offgel fractionator and a Bioanalyzer 2100 from Agilent Technologies (Santa Clara, CA, USA). A SIGMA 2K15 centrifuge (SIGMA Laborzentrifugen GmbH, Osterode, Germany) was used for protein extraction. A centrifuge Centromix (Selecta, Barcelona, Spain) was used to preconcentrate plankton samples. A Heidoph shaker, type Reax 2000, used for vortexing samples, was from Gemini B.V. (Apeldoorn, Netherlands). A Perkin Elmer Model 1100B (Perkin Elmer, Norwalk, CT, USA) atomic absorption spectrometer equipped with an HGA-700 graphite furnace atomizer, deuterium background correction and an AS-70 auto-sampler was used for determining Cd, Co, Cr, Cu, Mn, Ni, and Pb in the offgel fractions. The sources of radiation were monoelement hollow cathode lamps (HCLs) from Cathodeon, Cambridge, U.K. (Cr, Cu, Mn and Ni HCLs); whereas, Co and Pb HCLs were Lumina™ type from Perkin Elmer. An Optima 3300 DV inductively coupled plasma (ICP) atomic emission spectrometer (Perkin Elmer) equipped with an autosampler AS 91 (Perkin Elmer) and a Gem-Cone cross-flow nebulizer type (Perkin Elmer) was used for Cu, Fe and Zn determinations. A Hitachi double-beam spectrophotometer model U-2010 (Hitachi, Berkshire, UK) equipped with 10 mm quartz cells was used for all UV–visible measurements (Bradford assay).

All glass and plastic materials were rigorously cleaned and kept in 10% (v/v) nitric acid for at least 48 h. The material was then rinsed three times with ultra-pure water before being used. Finally, experiments regarding protein isolation from plankton as well as trace metal standard preparation were performed in a Class-100 clean fume hood (Telstar S.A., Tarrasa, Spain) to avoid contamination of samples and reagents.

2.3. Plankton sample collection

A fresh marine plankton sample (5 L) was collected in triplicate from a clam hatchery located in the Ría de Arousa estuary (Northwestern Spain). The phytoplankton sample consisted of a mixture of diatoms (*Skeletonema costatum*, *Chaetoceros gracilis*, *Chaetoceros calcitrans*, and *Chaetoceros muelleri*) and flagellate (*Tetraselmis chuii*, *Tetraselmis suecica*, *Isochrysis galbana*, *Isochrysis tahitian*, and *Monochrysis lutheri*). After collection, each plankton sample was centrifuged at 6400 *g* for 15 min at 20 °C. Because the centrifuge rotor consists of four 25 mL centrifuge tubes, several centrifugation stages were needed for obtaining plankton from the collected plankton sample (approximately 3 h when treating 1 L of plankton sample). After each centrifugation stage, the supernatant was removed, and the isolated plankton fractions