Microchemical Journal 124 (2016) 804–810

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Dissolved proteins characterization and speciation studies of metal-protein complexes in marine sediment pore water

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A R T I C L E I N F O

Article history:
Received 14 October 2015
Accepted 22 October 2015
Available online 30 October 2015

Keywords:
Dissolved proteins
Metal speciation
Marine sediment pore seawater
Tangential flow ultrafiltration
Offgel electrophoresis
Microfluidic lab-on chip electrophoresis

A B S T R A C T

Dissolved proteins characterization in marine sediment pore water has been performed by offgel electrophoresis (OGE) and microfluidic lab-on-chip (LOC) electrophoresis, assessing for the first time dissolved protein characteristics such as isoelectric point (pIs), and molecular weight (MW). In addition, speciation studies of metal-protein complexes in sediment pore water have also been performed by direct analysis of offgel fractions by electrothermal atomic absorption spectrometry (ETAAS), and inductively coupled plasma optical emission spectrometry (ICP-OES). Samples were subjected to a tangential flow ultrafiltration (UF) followed by a centrifugal UF (10 kDa molecular weight cut-off, MWCO) procedure, which offered a pre-concentration factor of 450. Isolation of dissolved proteins from the extracts has been performed by treating the ultrafiltrated fractions with methanol-chloroform as a precipitating reagent. The protein pellets were dried, dissolved in the offgel buffer, and subjected to optimized OGE and LOC electrophoresis procedures. Results showed proteins of MWs ranging from 5.1 to 87.0 kDa, and proteins exhibiting the highest MW were found in offgel fractions comprising pIs within the 6.34–7.76 range. Regarding metal-protein complexes, cadmium (Cd), and lead (Pb) were not found in any of the analyzed offgel fractions, which implies low affinity of these elements to the isolated proteins. However, iron (Fe) was found to be associated to proteins with pIs between 3.0 and 10 (the highest Fe concentrations were found in proteins from offgel fractions with pIs 6.3 to 7.7). Finally, copper (Cu), manganese (Mn), and zinc (Zn) were found to be bound to proteins exhibiting low MWs.

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1. Introduction

Dissolved organic matter (DOM) in sedimentary pore water comprises various kinds of organic compounds exhibiting high MWs [1]. Humic substances, and minor amounts of dissolved proteins, carbohydrates, and other non-humic macromolecules, are the components of higher MW (>1 kDa) [2]. Other substances such as fatty acids, sugars and amino acids (MW <1 kDa) are also present as lower molecular-weight compounds [2]. DOM plays a significant role in many physical and biogeochemical processes in aquatic environments, being an intermediate of the carbon flow rate, and participating in the sediment carbon preservation [1,2]. In addition, DOM in marine sediment pore water participates also in the transformation, availability and transport of organic and inorganic substances (anthropogenic pollutants such as trace metals), and affects therefore the flux of metal-complexing ligands from the sediment particles [3]. Therefore, the study of the DOM distribution/characterization of pore water has been a topic of interest, and some attempts have been performed over the past decades. However, because of the complex nature of DOM in the marine environment (sedimentary pore water included), most of the developments have been focused on chemical characterization of natural DOM based on its optical properties [4–9]. Developments on UV–visible absorption properties of colored DOM (CDOM) can be found in the literature for characterizing DOM in sedimentary pore water [4,5]. In addition, fluorescent properties of DOM (fluorescent DOM, FDOM) [4,6–9], mainly when applying three-dimensional excitation emission matrix fluorescence spectroscopy (3DEEM) [7,8], and 3DEEM combined with chemometric tools such as parallel factor analysis (PARAFAC) [4,9], have been shown to be the best way for DOM characterization on the basis of optical properties.

Few attempts based on MW distribution of DOM in pore water have been performed. As stated by Burdige and Komada [1], DOM in marine sediment pore waters has MWs less than ~3 kDa (~80–90% of total DOM in estuarine sediments and ~60–70% of total DOM in continental margin sediments), results that agree with other studies focused on characterizing DOM from nearshore marine and freshwater sediments. In these studies, UF procedures combined with size exclusion chromatography (SEC) have been used for assessing MW distribution of DOM.

Dissolved proteins are the most reactive substances belonging to marine DOM, and the assessment and characterization of these compounds in seawater have recently received special attention [10–16].

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http://dx.doi.org/10.1016/j.microc.2015.10.029
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However, no available data have been reported for the presence of protein in sedimentary pore waters. As previously stated, dissolved proteins must be present in sedimentary pore water at a very low concentration [2]. The use of several pre-concentration and separation stages before DOM determination/characterization is therefore needed. Tangential flow UF combined with centrifugal UF has been shown to offer an efficient concomitants removal, as well as a high pre-concentration rate of dissolved proteins from seawater [10–12]. Also for dissolved protein characterization in seawater, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional PAGE (2D-PAGE) have been proposed [10,13–16]. This technique presents, however, some drawbacks such as low sensitivity, and the need for tedious sample preparation stages for protein recovery from the gel. Risks of protein losses and sample contamination can therefore be occurred. These drawbacks are overcome when using OGE, for which proteins (pI-based fractionation) are recovered from the liquid phase. Possibilities of OGE as a preparative stage for multidimensional protein/peptides assessment (direct sampling of the isolated proteins/peptides into HPLC and electrophoresis instruments) have recently been reviewed [17]. Regarding the marine environment, OGE and LOC electrophoresis have recently be used for the assessment of protein in marine plankton and seawater [10,11,18] and also, for assessing trace elements in metal-protein complexes [11,19].

The aim of the current work has been the assessment of dissolved proteins in marine sediment pore water on the basis of their pIs and MWs. Developments on OGE and LOC electrophoresis after tangential flow and centrifugal UF methodologies have been performed for achieving low concentrations of dissolved proteins. An additional aim has also been focused on assessing metal-protein interactions by determining the amount of metals (Cu, Fe, Mn, Ni, and Zn) associated to the isolated proteins. This achievement has been performed by direct analysis of the offgel fractions by ETAAS and ICP-OES.

2. Experimental

2.1. Reagents

Ultrapure water, resistance 18 MΩcm, was obtained from a Milli-Q water-purification system (Millipore, Bedford, MA, USA). HAWP14250 (0.45 μm, 140 mm diameter) membrane filters (Millipore) were used for filtrating marine sediment pore water. Centrifugal UF was performed with Vivaspin 20 ultrafiltration tubes (size 0.6 m^2, polyethersulfone membrane of 10 kDa MWCO) from Sartorius Stedin Biotech (Goettingen, Germany). IPG Dry Strips (pH 3–10), offgel buffer 3–10, and Plus One DryStrip cover fluid used for pI protein fractionation were supplied by GE Healthcare Life Science (Uppsala, Sweden). The Protein offgel stock solution was prepared by dissolving 25.2 g of urea (electrophoresis grade, Sigma-Aldrich, St. Louis, MO, USA), 9.1 g of thiourea (analytical grade, Sigma-Aldrich), 600 mg of 1,4-dithiothreitol (DTT, electrophoresis grade, Merck, Darmstadt, Germany), 6 mL of glycerol solution (Merck), and 600 μL of offgel buffer (pH 3–10) in ultrapure water (final volume of 50 mL). Sizing and analysis of proteins by LOC electrophoresis were performed with the Agilent protein 80 kit (Agilent Technologies, Waldbronn, Germany). The kit consists of protein chips, Protein 80 Gel-Matrix and Protein 80 Dye Concentrate (filling gel for chips), Protein 80 Sample Buffer (preparation of the denaturing solution), Protein 80 Ladder [standard proteins with MWs of 6.5, 15, 28, 46 and 63 kDa (concentration of 600 ng μL^{-1} as a sum of all standard proteins)], and also, standard proteins coded as lower marker and upper marker (1 ng μL^{-1} and 60 ng μL^{-1}, respectively). Metal standard solutions were prepared from Cd, Cu, Fe, Mn, Ni, Pb, and Zn stock standard solutions (1000 g L^{-1}) from Scharlau (Barcelona, Spain). Other reagents were high purity 69% nitric acid, sodium azide, acetone, methanol, chloroform (Panreac, Barcelona, Spain); sodium hydroxide (Merck); ammonium hydrogencarbonate (BDH, Poole, UK); 2-mercaptoethanol, BME (Fluka, Vancouver, Canada); sodium dodecyl sulfate, SDS (AppliChem, Darmstadt, Germany); bovine serum albumin (Thermo Scientific, Rockford, IL, USA) M; and tris-(2-carboxyethyl)phosphine hydrochloride, TCEP, superoxide dismutase from bovine erythrocytes, SOD, and alcohol dehydrogenase from *Saccharomyces cerevisiae*, ADH (Sigma-Aldrich).

2.2. Instruments

The tangential flow UF system consisted of a Masterflex I/P pump (Millipore), a Prep/Scale-TFF Cartridge (Millipore) with a polyethersulfone membrane (nominal MW cut-off 10 kDa), and a Pre/Scale-TFF Holder (Millipore) equipped with a pressure gauge. Centrifugal UF was performed with an Alresa Digtor centrifuge (Madrid, Spain). A SIGMA 2K 15 centrifuge (SIGMA Laborzentrifugen GmbH, Osterode, Germany) was used when obtaining the protein pellets. Two dimensional electrophoresis was performed with a 3100 Offgel fractionator (Agilent Technologies, Santa Clara, CA, USA) and a Bioanalyzer 2100 with electrode cartridge for on-chip electrophoresis and fluorescence detection (Agilent). 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, Cu, Mn, Ni, and Pb bound to proteins of similar pIs (offgel fractions). The sources of radiation were monoelement hollow cathode lamps (HCLs): Cu. Mn, and Ni HCLs (Cathodeon, Cambridge, U.K.), and Lumina™ Pb HCL (Perkin Elmer). An Optima 3300 DV inductively coupled plasma-optical 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 Fe and Zn determinations.

2.3. Sample collection

Marine sediments were collected during the low tide at one site in an area of cockle (*Cerastoderma edule*) farming at Ría de Vigo estuary (north-western Spain). Sampling dates were from January 2013 to June 2013 at the same estuary (Ría de Vigo), although not always at the exact sampling site. For all cases, surface sediment was discharged, and marine sediment cores collected (10 cores close to each other) consisted of 0–30 cm in depth (approximately 20 cm in diameter). Sediments were mixed and stored in pre-cleaned plastic containers (20 mL capacity) which were hermetically closed. Once in the laboratory, the water accumulated at the top of the container during the transportation (about 2 h) was manually collected and decanted to a container for further filtration (0.45 μm). The sediment in the bottom of the container was subjected to centrifugation (4000 rpm, 4 °C, 20 min), and the extracted water was combined with the water obtained by natural decantation during the transport. Composite samples were filtered (0.45 μm) and immediately subjected to the tangential flow UF procedure.

2.4. Pore water tangential flow UF

Before treating samples, the tangential flow UF system was cleaned by passing 2 L of 0.1 M NaOH at 45 ± 5 °C (this solution was recirculated for 60 min), followed by 9 L of Milli-Q water, also at 45 ± 5 °C. Operating conditions for the tangential flow UF process were based on the procedure described by García-Otero et al. when pretreating seawater [20] with slight modifications. Filtered pore seawater samples (9 L) were treated with 0.325 g of sodium azide (final concentration of 5.0 mM in pore water) and 0.1 g of SDS (final concentration of 0.01%(m/v)) to prevent protein adsorption onto the UF membrane. Pore water samples were then concentrated until obtaining a volume of retentate within the 150–300 mL range.

2.5. Retentate centrifugal UF

Before centrifugal UF, the ultrafiltration tubes were cleaned (glycerine removal from the polyethersulfone membrane) with 20 mL of

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