Environmental and Experimental Botany 147 (2018) 63–74

Contents lists available at ScienceDirect

Environmental and Experimental Botany

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

Proteomic analyses of the cyanobacterium *Arthrospira* (*Spirulina*) *platensis* under iron and salinity stress

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ARTICLE INFO

Keywords:
Abiotic stress
Iron
Salinity
2D electrophoresis
Growth
Pigments

ABSTRACT

Arthrospira platensis is a species of filamentous cyanobacteria whose high protein content renders it suitable as a nutritive supplement for human and animal diets. Furthermore it is easy to grow as it tolerates a broad range of environmental stresses such as extremes of pH, salinity and heavy metals. In this study, changes in the growth, pigments and proteome of *A. platensis* were analyzed under two major abiotic stresses limiting productivity, salinity and iron. The lower concentrations of stresses showed varied effects on fresh weight, growth rate, pigment constituents and soluble protein of *A. platensis*. A proteomic analysis was conducted using SDS-PAGE first to obtain an overview of gross changes, followed by 2-DE and mass spectrometry to identify specific proteins whose abundance was affected by these environmental stresses. Eighteen spots were differentially expressed under the stress conditions. Of these, six were found with increased abundance responding only to Fe^{2+} stress, and five as a result of NaCl stress alone. Using LC–MS/MS, 37 proteins were identified within these spots. These proteins were classified into thirteen categories according to their function annotation.

The present analysis could provide important data related to stress-tolerance by microalgae.

1. Introduction

Arthrospira platensis is a filamentous blue-green alga from a genus that has been commercially cultivated to produce food supplements for human diets and animal feed e.g. for fish and shrimp (Vonshak, 1997a). It is a rich source of protein (60–70% of its dry weight; Spolaore et al., 2006), vitamins, minerals, essential fatty acids, carotenes, pigments and other components with antioxidant activity (Vonshak, 1997b; Lin et al., 2007). Various species of *Arthrospira* have been isolated from a wide range of aquatic environments and they have been found to adapt well to extreme conditions. Their habitats include brackish water, sea water, freshwater ranging from subarctic waters to tropical lagoons and even hot springs. Its alkalophilic capacity (i.e. abundant growth at pH as high as 11.0) is also a remarkable example of this species' unique adaptability to extreme environments (Priya Sethu, 1996). *Arthrospira* spp. are also well suited for other industrial purposes, e.g. for production of useful metabolites (Hongsthong et al., 2008; Ismaiel et al., 2014) and for basic scientific research – for example they are providing essential data concerning protein folding, membrane properties and repair mechanisms (Rothschild and Mancinelli, 2001).

Abiotic stress factors have received much attention in the last decades for many reasons. Chief among these are increasing environmental hazards resulting from climate change, and soil depletion resulting from agricultural activity – especially increasing soil salinity from prolonged irrigation (Ondrasek et al., 2011). Salinity or salt stress is an increasing concern in many parts of the world that depend on intensive agricultural production (Gao et al., 2011). Metal contamination of soils also poses risks, even though some metals such as iron are essential micronutrients and are required for the proper growth for all organisms. High concentrations of these elements can impose abiotic stress to algal cells (Bruland et al., 1991; Ismaiel et al., 2014). Iron is one of the most abundant metal elements on earth (McDonald et al., 2010). It is an essential microelement for all living organisms (Rout and Sahoo, 2015) because it is a constituent of many functional proteins molecules such as cytochromes, catalase, and superoxide dismutase, and in photosynthetic organisms such as *A. platensis* it is required to synthesize and maintain chlorophyll (Ismaiel et al., 2014; Rout and Sahoo, 2015). However, over-exposure to iron causes oxidative stress leading to homeostatic imbalances of the intracellular redox state which can lead to cellular damage and even death if it is left uncontrolled (Rout and Sahoo, 2015). Detoxification mechanisms to prevent or reduce the effects of heavy metal accumulation have been

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https://doi.org/10.1016/j.envexpbot.2017.11.013
Received 20 July 2017; Received in revised form 28 October 2017; Accepted 24 November 2017
Available online 26 November 2017
0098-8472/

widely reported. Intracellular levels of iron (as Fe^{2+} and Fe^{3+}) are carefully regulated by iron-carrier proteins such as phytochelatins (PCs) and metallothioneins (Cobbett and Goldsbrough, 2002) which bind heavy metals and thus prevent the toxic action of heavy metal ions in the cytoplasm.

Ferritin is another important iron-carrier protein which stores iron and controls its release upon demand, hence reducing its potential damage to cells (Komatsu et al., 2014). Ferritin levels have also been reported to be increased significantly in crops in response to salt stress, together with glutathione S-transferases (GSTs) which are involved in scavenging of reactive oxygen species (ROS) produced as a result of the imposed abiotic stress (Komatsu et al., 2014).

Although comparative proteomics analyses have been reported from *Arthrospira* responding to different types of abiotic stresses, including low- and high-temperature (Hongsthong et al., 2008; Jeamton et al., 2008) and salinity (Wang et al., 2013), very little is known about salt-responsive proteins or the molecular mechanisms of salt tolerance in *Arthrospira*. In addition, the regulation and expression of these proteins still need further examination. The main purpose of the present study was to perform physiological and proteomic analyses of *A. platensis* in response to iron and salinity stresses using multi-dimensional liquid chromatography and tandem mass spectrometry. Moreover, to emphasis on some specifically expressed proteins for further exploiting of their role in adaptability of cyanobacteria.

2. Materials and methods

2.1. Organism and culture conditions

Arthrospira platensis (Gomont) Geitler (MIYE 101) was obtained from the Phycology Lab, Faculty of Science, Zagazig University, Egypt. *A. platensis* was cultured as described by Zarrouk (1966). The stress concentrations were chosen following a preliminary screen as described previously (Ismaiel et al., 2014). Iron concentrations ranged from 0.09 to 1 mM Fe ($FeSO_4$-chelated, in a molar ratio of 1:2 Fe: Na_2-EDTA); while the salinity concentrations ranged from 42.5 to 340 mM NaCl. The standard concentrations in control medium were 0.035 mM Fe^{2+} and 17 mM NaCl.

Following inoculation with a mid-log phase culture (approx. 0.7 mg dry weight), flasks were grown at 31 ± 0.5° C while illuminated with cool white fluorescent lights (Duro-Test 20 W Vita-Lite) at 60 µmol photons $m^{-2}\,s^{-1}$ for 14 days. The cultures were shaken once daily by hand. Cells were harvested by centrifugation at 6,000 *xg* for 10 min at 4 °C, (Sorvall Legend X1R, Thermo Scientific) and washed with 10 mM Na_2-EDTA, and with distilled water, then frozen in liquid nitrogen, and stored at −80° C until required.

2.2. Growth and pigment analyses

Specific growth rate (µ) measurements were conducted as described previously (Vonshak, 1997b). Chlorophyll *a* (Chl *a*) and carotenoids contents were quantified as described by Metzner et al. (1965) with minor modifications (Ismaiel, 2016). The concentration of C-phycocyanin (C-PC) was calculated according to the method of Bennett and Bogorad (1973).

2.3. Protein extraction and quantification

Total proteins were extracted from the algal pellets using an acetone/trichloroacetic acid (TCA) precipitation method (Damerval et al., 1986). Briefly, 0.4–0.9 g fresh weights (FW) equivalent of algal pellets were used for protein extraction. Samples were homogenized with an equal volume of glass beads (0.45–0.50 mm in diameter) in glass centrifuge tubes containing 1 ml of pre-chilled acetone and 10% (w/v) TCA, 0.07% (w/v) dithiothreitol (DTT) and kept at −20° C overnight. The samples were centrifuged at 10,000 *xg* for 20 min, at −5° C. The supernatants were discarded and the pellets were washed with pre-chilled acetone/0.07% (w/v) DTT and stored for 30 min at −20° C. This washing step was repeated seven times.

The final pellets were dried carefully under a gentle stream of nitrogen gas to remove all acetone and re-dissolved in IEF solution (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT and 0.5% (v/v) of pH 3–10 ampholyte (Bio-Rad Laboratories, Hercules CA, USA), as described previously (Rampitsch and Bykova, 2009). In preparation of IEF, the protein content of each sample was determined using a Bradford assay (BioRad), and a volume of 450 µl of each sample was adjusted to contain 550 mg protein (BSA equivalents).

2.4. 2-DE (IEF/SDS-PAGE) and gel analysis

Two-dimensional electrophoresis was carried out as described by Rampitsch and Bykova (2009). In brief, IPG strips (24 cm, pH 4–7, Immobiline DryStrip, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were rehydrated passively and separated by IEF in a Multiphor II unit (GE Healthcare) for a total of 58 kVh. Strips were then equilibrated (reduced and alkylated) as recommended by the manufacturer and transferred to 12% SDS-PAGE gels for the second dimension (Ettan Dalt-6, GE Healthcare). Electrophoresis was carried out with a 0.5 W gel^{-1} for the first hour followed by a 17 W gel^{-1}.

The gels were fixed in 12.5% w/v TCA, 30 min and stained overnight with CBB G-250. After destaining in distilled water, gels were scanned (Epson Expression 1680, Epson, CA, USA) to produce TIF images and then printed for visual analysis. The 2-DE gels were prepared from six independent biological replicates for each treatment. Only consistent differentially expressed protein spots were processed for identification using LC-ESI–MS/MS. Spot intensities were quantitated manually by using ImageJ software (http://rsb.info.nih.gov) as described by Natale et al. (2011). The selected protein spots were excised from the 2-DE gels, and digested in-gel using trypsin (Sequencing grade modified trypsin, Promega, Madison, WI, USA) as previously described (Shevchenko et al., 1996).

2.5. Statistical analysis

All measurements were done in triplicates and the data were represented as mean (± standard deviation, SD). The SPSS 10.0 software (SPSS, Richmond, VA, USA) was used for statistical calculations. One-way analysis of variance (ANOVA) with Duncan's multiple range tests was used for comparison of the significance level between values at $P < 0.05$.

2.6. Mass spectrometry and data analysis

Mass spectrometric analysis by LC-ESI–MS/MS was conducted as described by El-Bebany et al. (2010). Briefly, a nanoflow HPLC system (Ultimate 3000: Dionex ThermoFisher, Bremen, Germany) was used to separate peptides using a standard acetonitrile gradient on a C_{18} column (5 µm particle size, 300 Å pores, 10 cm) eluting directly into a linear ion trap mass spectrometer (LTQ XL: Thermo Fisher Scientific, Inc., San Jose CA, USA) at 250 $nl.min^{-1}$ via nano electrospray ionization. A "Top 10" programme was used for MS/MS analysis: the ten most abundant peaks in each survey scan were used as precursor ions for an MS^2 spectrum, with dynamic exclusion for 30 s. After acquisition, raw spectral data files were converted to MGF using Mascot Distiller (v2.0, MatrixScience, London UK) and queried against the NCBI non-redundant database (14.48 million entries). The following parameters were included with each search: trypsin with one missed cleavage permitted; fixed modification of C (cam), variable modifications of M (ox) and NQ (deam); the precursor tolerance was ± 1 Da, MS/MS tolerance was ± 0.8 Da. Automatic decoy searches were included with each query.