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RESEARCH NOTE

Methylglyoxal metabolism in seaweeds during desiccation

Metabolismo de metilglioxal en macroalgas durante desecación

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Abstract. In primary producers, diverse stressors cause an over-production of methylglyoxal (MG), which is principally detoxified by glyoxalase I (GLO1) activity. A recent proteomic study found that GLO1 was up-regulated during natural desiccation in the red seaweed *Pyropia orbicularis*, which inhabits the upper intertidal rocky zone and compared to other species, is highly tolerant to air exposure. To better understand and determine differential responses to desiccation stress, this study evaluated MG concentration and GLO1 activity in two species with contrasted vertical distribution, *P. orbicularis* and *Lessonia spicata* (lower distribution). Results showed that *P. orbicularis* successfully scavenges MG via increased GLO1 activity during desiccation. In contrast, GLO1 activity in *L. spicata* did not increase during desiccation, resulting in MG overproduction. This study is the first to quantify MG and GLO1 levels in seaweeds during natural stress, and partly explain the mechanisms by which *P. orbicularis* is dominant in the upper rocky intertidal zone.

Key words: Methylglyoxal, glyoxylase I, *Pyropia*, *Lessonia*, desiccation

INTRODUCTION

Methylglyoxal [MG; 2-oxopropanal (CH₃COCHO)] is a cytotoxic compound formed by the fragmentation and elimination of phosphate from the phosphoenolpyruvate form of glyceraldehyde-3-phosphate (GA3P) and dihydroxyacetone-phosphate (DHAP) (Thornalley 1996). It can also be produced from aminoacetone (Sartori *et al.* 2008) and an ozone reaction with aromatic hydrocarbons (Grosjean *et al.* 1996). MG is both a mutagenic and genotoxic compound, since it can degrade proteins, inactivate the antioxidant defense system, and provoke cellular death (Martins *et al.* 2001) by altering macromolecules through the modification of advanced glycation end products (Hossain & Teixeira 2011). During cellular stress, the rate of glycolysis increases in diverse organisms (Wu & Juurlink 2002, Yadav *et al.* 2005, Singla-Pareek *et al.* 2006, Kumar *et al.* 2013), leading to an imbalance that causes a spontaneous MG overproduction (Hossain *et al.* 2009). In primary producers, if overproduced MG is not detoxified immediately after its production in the chloroplast, it will act as an intrinsic mediator that catalyzes the photoreduction of O₂ at PS I, leading to the production of O₂•⁻, which generates oxidative stress (Saito *et*

al. 2011). Efficient MG detoxification during normal physiological processes or when under various stressors is one of the adaptive stress tolerance strategies of plants (Hossain & Teixeira 2011). Indeed, most organisms protect themselves from the deleterious effects of MG by detoxifying it through the glyoxalase pathway, which is comprised of 2 enzymes: *glyoxalase I* (GLO1) (lactoylglutathione lyase; EC 4.4.1.5), which uses GSH as a cofactor for the conversion of MG to S-D lactoylglutathione, and *glyoxalase II* (GLO2) (hydroxyacyl glutathione hydrolase; EC 3.1.2.6), which gives GSH back to the system, leading to the production of D-lactate (Hossain *et al.* 2009).

GLO1 activity is not stress-specific but is activated under several environmental conditions (Blomstedt *et al.* 1998, Hossain *et al.* 2009, 2010). In pumpkin seedlings, GLO1 activity increases under conditions of drought, salinity, and white light, among others (Hossain *et al.* 2009). Additionally, mung beans up-regulate glyoxalase pathway enzymes through glycinebetaine- and proline-increased tolerance to cadmium stress (Hossain *et al.* 2010). In *Sporobolus stapfianus*, a

desiccation-tolerant plant, GLO1 transcripts increase as relative water content diminishes (Blomstedt *et al.* 1998).

Desiccation is a recurrent environmental stress for seaweeds from the intertidal rocky zone, such as *Pyropia orbicularis* (Rhodophyta, Bangiales) commonly known as luche or nori (Ramírez *et al.* 2014, Guillemín *et al.* 2016). This species is frequently exposed to air during normal tide cycles in nature (Contreras-Porcia *et al.* 2011, 2012). A series of studies using physiological, biochemical, transcriptomic, and proteomic approaches have been conducted on *P. orbicularis*, with data revealing the mechanisms that allow this species to reside in the upper intertidal rocky zone, where desiccation stress is more severe than lower intertidal zones (Contreras-Porcia *et al.* 2012, 2013; Flores-Molina *et al.* 2014). Using a proteomic approach, López-Cristoffanini *et al.* (2015) found for the first time that GLO1 is up-regulated during desiccation in seaweeds, suggesting an enzymatic control of MG levels during tidal fluctuations. Given these findings, it has been hypothesized that the control of MG levels in *P. orbicularis* is part of the tolerance mechanisms employed by this species to grow and survive in the upper intertidal rocky zone, contrary to many other algal species (López-Cristoffanini *et al.* 2013, Flores-Molina *et al.* 2014, Guajardo *et al.* 2016). Therefore, the objectives of this study were to determine MG levels and GLO1 activity in the red alga *P. orbicularis* during the desiccation-rehydration cycle and to compare these responses with those of *Lessonia spicata* (Ochrophyta, Laminariales), a desiccation-sensitive species (Flores-Molina *et al.* 2014) with a lower intertidal distribution.

MATERIALS AND METHODS

Naturally hydrated fronds of *P. orbicularis* and *L. spicata* were collected and transported to laboratory as in Contreras-Porcia *et al.* (2011). Additionally, tolerance to the daily desiccation-rehydration cycle was assayed *in vitro* under 1 and 4 h of desiccation and 2 and 4 h of rehydration; and fronds were kept at -32°C until later use (Contreras-Porcia *et al.* 2011). For *in vitro* desiccation, plants were initially blotted dry and exposed to air in a growth chamber during 4 h at 12°C and with an irradiance of 70-80 $\mu\text{m photon m}^{-2} \text{s}^{-1}$. Following desiccation, a subset of dehydrated fronds was immediately rehydrated in 0.22 μm of filtered seawater for 4 h to characterize the recovery from oxidative stress.

The MG levels and GLO1 activity of both species were measured in all collected fronds [natural hydration, *in vitro* desiccation (1 and 4 h), and rehydration (2 and 4 h)]. For MG extraction, 0.5 g of dry tissue (DT) samples, were frozen in liquid nitrogen, ground with a mortar and a pestle, homogenized on ice for 15 min with 3 mL of 0.5 M perchloric acid, and

centrifuged at 11,000 x g for 10 min at 4°C. The supernatant was decolorized for 15 min at room temperature by adding 10 mg mL⁻¹ charcoal, centrifuged at 11,000 x g for 10 min, and neutralized with a saturated solution of potassium carbonate at room temperature for 15 min, followed by centrifugation at 11,000 x g for 10 min. The supernatant was frozen at -32°C until spectrophotometric and chromatographic determinations of MG were performed.

Pure MG showed low radiation absorption, as determined by spectrophotometry. Therefore, a derivatization with 1,2-diaminobenzene was performed in order to obtain a high absorbance molecule, 2-methylquinoxaline, as described in Yadav *et al.* (2005). For this, different concentrations (5-100 $\mu\text{mol L}^{-1}$) of pure MG (Sigma-Aldrich, USA) were derivatized, and 2-methylquinoxaline was measured by spectrophotometry with a UV/Visible Smartspect 3000 (BioRad, USA) at a wavelength range of 200-700 nm to determine the maximum value in its absorption spectra. A 2 mL reaction mixture containing 0-100 $\mu\text{mol L}^{-1}$ MG, 1.98 mmol L⁻¹ 1,2-diaminobenzene, 0.5 mol L⁻¹ perchloric acid, and double-distilled water was used. Additionally, MG concentration was assessed by high performance liquid chromatography coupled to UV detection (HPLC/UV) (Shimadzu, Kyoto, Japan), and determined using a wide range standard curve (0.2-50 $\mu\text{mol L}^{-1}$) of pure MG that obeys the Beer-Lambert Law. The 2 mL reaction mixture contained 0.2-50 $\mu\text{mol L}^{-1}$ MG or the algal extracts, 1.98 mM 1,2-diaminobenzene, 0.5 M perchloric acid, and double-distilled water. A 20 μL aliquot was injected onto an HPLC column (Inertsil ODS-3 RP-C₁₈ 4.6 x 250 mm, 5 μm , GL Sciences, USA) using an isocratic mobile phase consisting of 25 mM ammonium formate buffer (pH 3.4) and methanol 60:40% v/v, with a 1 mL min⁻¹ flow. Derived MG was detected at 334 nm.

To quantify GLO1 activity, proteins from algal tissue samples were extracted and quantified according to Contreras *et al.* (2005). GLO1 activity was measured by spectrophotometry following the initial rate of increase in absorbance at 240 nm, as described in Arai *et al.* (2014) with slight modifications. Briefly, a reaction mixture containing 0.7 mM glutathione (GSH), 0.7 mM MG, and 50 mM sodium phosphate buffer (pH 6.6) was incubated for 10 min at 37°C. Subsequently, 40 μg of protein extract was added to complete a total volume of 2 mL, and absorbance was continuously monitored for 5 min at 240 nm. For each sample, a non-enzymatic control was included, and GLO1 activity was calculated using the change in molar extinction coefficient between the enzymatically generated s-lactoylglutathione and the spontaneous reaction between MG and GSH that forms a hemithioacetal ($\epsilon = 2.86 \text{ mM}^{-1} \text{cm}^{-1}$).

RESULTS AND DISCUSSION

Since MG in algal tissue samples could not be directly identified by spectrophotometric methods due to low radiation absorption, it was derivatized to a quinoxaline with a strong UV absorption band, as described by Yadav *et al.* (2005). This substantially improved quantitative determinations by spectrophotometry at low MG concentrations. Yadav *et al.* (2005) propose that the maximum absorption of derivatized methylglyoxal detected by HPLC/UV is at 320 nm, but previous spectrophotometric assays have shown variable absorbance data (Cordeiro & Freire 1996). Therefore, an absorbance curve between 200-700 nm was performed, and maximum absorption at a low concentration (10 μM) was determined to be at 334 nm (Fig. 1). This wavelength was used to quantify MG in the seaweeds subjected to desiccation stress. Furthermore, HPLC/UV and spectrophotometric methods showed acceptable selectivity since extracts without MG did not show detectable signals (results not shown). Additionally, HPLC methodology was optimized to determine MG content in algal extracts (absolute calibration curve of 1-50 $\mu\text{mol L}^{-1}$) (Fig. 2), where the detection and quantification limits were $6 \times 10^{-4} \mu\text{mol L}^{-1}$ and $2 \times 10^{-4} \mu\text{mol L}^{-1}$, respectively, with low error.

At a biological level, a slight increase of MG in *P. orbicularis* occurred during desiccation (60 $\text{nmol g}^{-1} \text{DT}$); however, these levels were not significant (ANOVA, $T=0.420$, $P=0.992$) in comparison with naturally hydrated plants (55 $\text{nmol g}^{-1} \text{DT}$) (Fig. 3A). During rehydration (2 and 4 h), the MG levels were lower than in desiccated tissue, but similar ($T=2.53$, $P=0.160$; $T=2.45$, $P=0.179$, respectively) to those registered during maximum natural hydration (higher after 8 h of high tide hydration). In contrast, the MG levels in *L. spicata* increased significantly ($T=5.74$, $P=0.0014$) during desiccation, from 37 $\text{nmol g}^{-1} \text{DT}$ registered during hydration to 100 $\text{nmol g}^{-1} \text{DT}$ after 4 h of

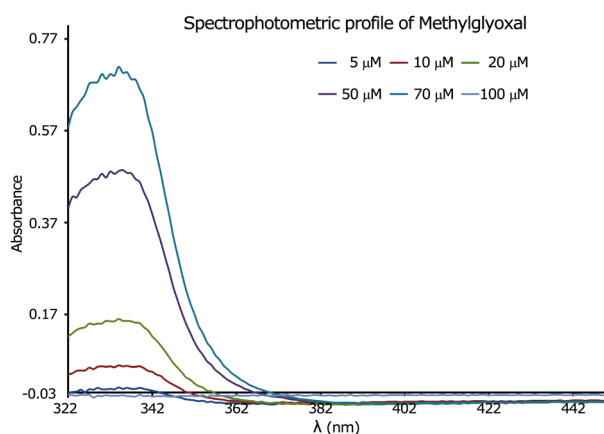


Figure 1. Absorption spectra for a range of concentrations of MG / Espectro de absorción para un rango de concentraciones de MG

desiccation (Fig. 3A). While these levels dropped 45-50% during rehydration, the values registered during maximum hydration were not recovered ($T=6.613$, $P<0.001$) due a low MG detoxification.

GLO1 activity increased significantly during desiccation in comparison to hydration ($0.5 \text{ pg min}^{-1} \mu\text{g}^{-1} \text{protein}$) in *P. orbicularis* (Fig. 3B), with maximum specific activity after 4 h of desiccation ($4 \text{ pg min}^{-1} \mu\text{g}^{-1} \text{protein}$) ($T=15.99$, $P<0.001$). This activity decreased by 38% after 4 h of rehydration, but did not reach basal levels ($1.5 \text{ pg min}^{-1} \mu\text{g}^{-1} \text{protein}$; $T=4.97$, $P=0.004$). In *L. spicata*, a slight increase in GLO1 activity was determined after 1 h of desiccation, going only from $0.3 \text{ pg min}^{-1} \mu\text{g}^{-1} \text{protein}$ during hydration to $1 \text{ pg min}^{-1} \mu\text{g}^{-1} \text{protein}$ during desiccation ($T=9.63$, $P<0.001$). No increased activity was observed during rehydration (Fig. 3B).

These data provide evidence of an over-production of MG in the desiccation-sensitive species *L. spicata*, concomitant with low GLO1 activation. In contrast, GLO1 had active participation during desiccation stress in *P. orbicularis*, which explains the attenuated MG production that occurs during the natural desiccation-rehydration cycle. The high levels of MG in *L. spicata* demonstrate a low scavenging of this toxic compound, suggestive of high cellular oxidation. In fact, higher lipid and protein oxidation occurs in *L. spicata* tissue during desiccation than in *P. orbicularis* (Contreras-Porcia *et al.* 2011, Flores-Molina *et al.* 2014, Guajardo *et al.* 2016), which impedes cellular stability during extended air exposure. To our knowledge, this is the first study showing GLO1 activity in a seaweed species, and the high activity of GLO1 observed in the desiccation-stressed fronds of *P. orbicularis* provides additional insights of desiccation tolerance of seaweeds to inhabit the upper intertidal rocky zone.

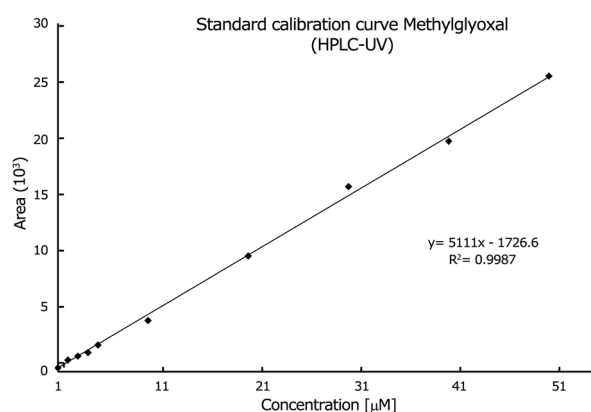


Figure 2. Absolute standard calibration curve obtained for determination of 2-methylquinoxaline, derivatized MG, by HPLC/UV at 334 nm. Values are mean \pm SD of 5 replicates / Curva de calibración estándar absoluta obtenida para la determinación del derivado de 2-metilquinoxalina de MG por HPLC/UV a 334 nm. Los valores son la media \pm DS de 5 réplicas

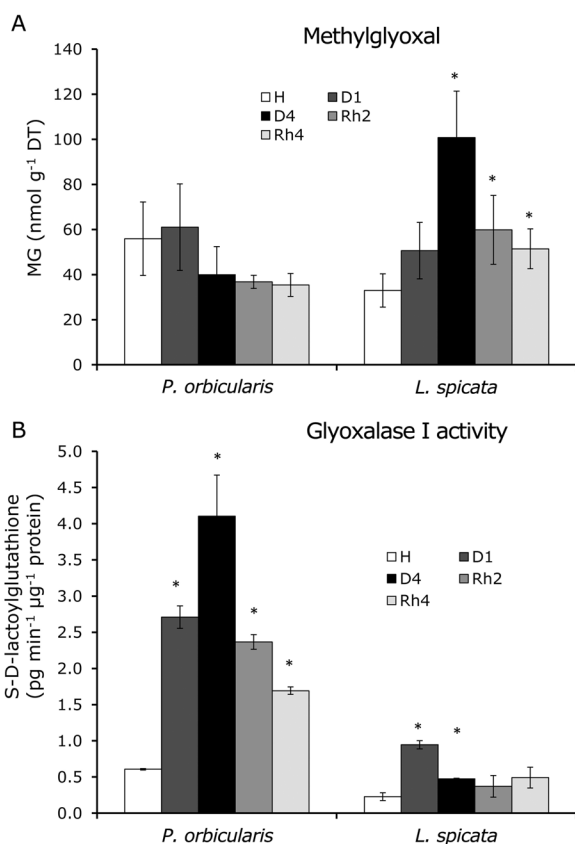


Figure 3. MG concentration (expressed in $\text{nmol g}^{-1} \text{DT}$) (A) and specific activity of GLO1 (expressed as S-D-lactoylglutathione $\text{pg min}^{-1} \mu\text{g}^{-1} \text{protein}$) (B) in *P. orbicularis* and *L. spicata* during a desiccation (D)-rehydration (Rh) cycle. The asterisk above the histogram indicates significant differences with hydration at $P < 0.05$. DT, dry tissue. Values are mean \pm SD of 5 replicates / Concentración de MG (expresada en $\text{nmol g}^{-1} \text{DT}$) (A) y la actividad específica de GLO 1 (expresada como proteína S-D-lactoilglutathione $\text{pg min}^{-1} \mu\text{g}^{-1}$) (B) en *P. orbicularis* y *L. spicata* durante un ciclo de desecación (D)-rehidratación (Rh). El asterisco arriba del histograma indica diferencias significativas con hidratación $P < 0,05$. DT, tejido seco. Los valores son la media \pm DS de 5 réplicas

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