Glutathione and zeaxanthin formation during high light stress in foliose lichens

J. Štepigová, H. Vráblíková, J. Lang, K. Večeřová, M. Barták

Institute of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic

ABSTRACT

In the presented study, we describe techniques for glutathione and pigment determination in lichens used in our laboratory. Glutathione and xanthophyll cycle pigments, especially zeaxanthin, are important antioxidants protecting plants against various stresses. In our laboratory, the high light stress in lichens has been intensively studied for several years. We extract glutathione in HCl and determine it by thiol-binding fluorescence label monobromobimane. For pigment determination, homogenized lichen thalli are extracted with pure acetone. According to our results, the total amount of glutathione decreases after a short-term high light exposure, while the amount of zeaxanthin increases.

Keywords: antioxidants; glutathione; high light stress; lichens; xanthophyll cycle; zeaxanthin

Reactive oxygen species (ROS, e.g. $^1O_2$, $O^{\cdot}$, O_3) occur within plant tissues as a consequence of various stresses. Since they are very unstable, they can damage cell structures. They react with a wide range of cell components, such as pigments and proteins (Bhatacharjee 2005). To avoid cell damage, plants possess several mechanisms that deactivate ROS. Antioxidants (AO) represent one of the protective mechanisms against harmful ROS; glutathione, ascorbate, pigments of xanthophyll cycle and tocopherol belong among the most important of them (Blokhina et al. 2003). Besides higher plant tissues, the AO are also present in green algae, which form lichen thalli in symbiosis with lichenized fungi. In plants and lichens, ROS formation leads to changes in amount and redox state of AO. Therefore, the investigation of AO dynamics under various stresses allows us to quantify the capacity of protective mechanisms in plant cells. In a majority of plants, the total amount of glutathione increases during, or immediately after, a long-term stress (Noctor and Foyer 1998). In a short-term stress, the opposite response is reported as documented for instance for shade leaves of *Begonia* after their transfer to full sunlight (Burritt and MacKenzie 2003). A similar rapid decrease in total glutathione was observed during a short-term pathogen attack (Vanacker et al. 2000) and drought stress (Loggini et al. 1999). The turnover of glutathione in plants under long-lasting stress reflects both short- and long-term response of glutathione. During stress, redox state of glutathione is altered as well. It was shown (e.g. Barták et al. 2004) that the ratio of oxidized to reduced glutathione increased after the treatment of thalli with high light. In lichens, the majority of studies focused on glutathione dealt with its dynamics during thallus desiccation (e.g. Kranner and Birtic 2005, Weissman et al. 2005). An involvement of glutathione in high light stress in lichens has been studied recently (Kranner et al. 2005).

Zeaxanthin (Z) is a xanthophyll cycle pigment present in thylakoid membranes. It plays an important role in a dissipation of an excess light energy. It is formed from a diepoxide violaxanthin (V) via monoepoxide antheraxanthine (A). The deepoxidation of V to Z requires the presence of light; V recovers from Z in dark. There are several hypotheses trying to describe Z photoprotective

This contribution was presented to the 4th Conference on Methods in Experimental Biology held in 2006 at the Horizont Hotel in Šumava Mts., Czech Republic.

mechanism. It is likely that Z acts as a direct acceptor of energy from excited chlorophyll *a* in a reaction centre of photosystem II (Frank et al. 1994). Havaux et Nyiogi (1999) suggested that Z interacts and deactivates ROS. Another hypothesis postulates that Z changes conformation of light-harvesting complexes, and thus results in an enhanced thermal dissipation of excess energy (e.g. Gilmore 1997).

MATERIAL AND METHODS

Lichen collection and handling. Lichens are poikilohydric organisms. Their collection and storage differ from that used for homoiohydric plants. We investigated foliose species *Lasallia pustulata* and *Umbilicaria antarctica* collected from different locations (Ketkovice near Brno in the Czech Republic, Galindez Island in maritime Antarctica, respectively). Thalli of the above lichen species were transferred to a laboratory in Brno, air-dried and stored in a temperature-controlled room at 5°C under dim light (10 μmol/m^2/s of photosynthetically active radiation – PAR). Before experiments, thalli were rewetted with demineralized water under dim light at 15°C for 24 hours. Throughout the experiments, the thalli were kept in a fully hydrated state by regular spraying with demineralized water.

Set up for a photoinhibitory experiment. To quantify the effect of high light (HL) on the content of AO in thalli, we exposed lichens to 1500 and 1800 μmol/m^2/s PAR for 30 minutes. A halogen lamp was used as a source of homogenous light. Excess heat was eliminated by water filter with circulating water placed between the light source and exposed lichen thalli. Throughout the experiments, the thalli were kept in a fully hydrated state by contact of lower thalli surfaces with wet filter paper, and by regular spraying with demineralized water. Constant thallus temperature and air relative humidity were controlled by temperature and air humidity sensors connected to the data logger HOBO (Onset Computers, USA).

Sample preparation. Segments of lichen thalli (typically 80–100 mg dry weight for glutathione and 50–80 mg dw for pigment extraction) were taken regularly during high light experiments. They were immediately frozen in liquid nitrogen. After freeze-drying (Heto Maxi Dry Lyo, Denmark), dry thalli segments were homogenized using a ball mill (Retsch ZM 100, Germany). Dry powder was used for extraction of glutathione and pigments as specified below.

Glutathione. In lichens, three different methods are used for determination of glutathione and glutathione disulphide (Kranner 1998). In our study, a method with labeling thiols with monobromobimane (mBBr) was used. The method allows determination of both oxidized (GSSG) and reduced (GSH) glutathione. Dry lichen powder was extracted with 2 ml 0.1M HCl with addition of 60 mg of polyvinylpyrrolidone (PVP). Lichens contain phenolic secondary metabolites that can interact with glutathione during extraction. PVP binds phenolic compounds and eliminates their negative effect on reactions. The extract was divided into 2 parts. Total glutathione (GSH + GSSG) was determined in the first part; in the second part of the extract, thiol groups of GSH were blocked using N-ethylmaleinimid (NEM), which allowed to determine the amount of GSSG only. Disulphidic groups (-S-S-) in both parts of original extract were reduced by dithiotreitol (DTT) during the following step. Thiol groups were labeled with excess of monobromobimane (mBBr); approximately 1 μl of mBBr per 6 μl of extract was added. Samples incubated 15 minutes at room temperature. Then, 0.25% methanesulfonic acid (approximately 2.2 μl per 1 μl of extract) was added to acidify the sample. This step allows detection of mBBr using a fluorescence detector. GSH and GSSG were separated from low molecular thiols present in the extract and quantified by a reverse-phased HPLC (Figure 1). We used HPLC (Separation module 2690, Waters, USA) with fluorescence detector (Shimadzu RF-535), and separation column C_{18}. We used separation protocol described in Kranner (1998).

Xanthophyll cycle pigments. Pigments were extracted according to Pfeifhofer et al. (2002) with slight modifications as follows. We used 100% acetone as an extraction medium. Instead of mortar and pestle, as used in Pfeifhofer et al. (2002), we added small glass balls to lichen powder (see Sample preparation) for more effective homogenization. Small amount of $CaCO_3$ was added to lichen powder to eliminate secondary metabolites, lichen acids in particular. Chilled acetone was added into a microtube with lichen powder, shook vigorously (VORTEX) for few seconds and centrifuged at 4°C for 10 min at 36 000 g. Supernatant with extracted pigments was saved. The process was repeated 3–4 times. Pigments were separated by HPLC (Separation module 2690, Waters, USA) using photodiode-array detector (DAD 996,

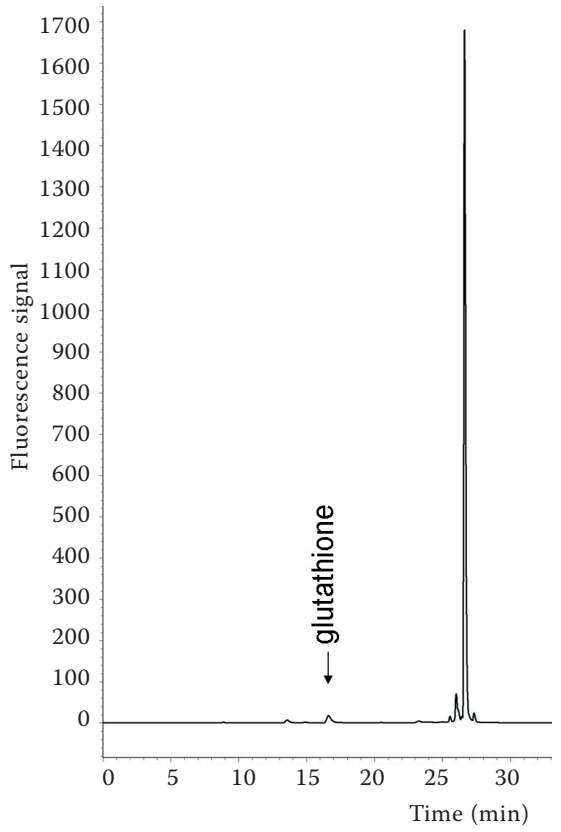

Figure 1. Typical HPLC chromatogram of thiol separation in *L. pustulata*

Waters, USA). Solvent A consisted of acetonitril, methanol and 0.1M Tris-HCl buffer (72:8:3). Solvent B contained methanol and hexane (4:1). Linear gradient started with 100% of solvent A. Its volume decreased to 0% within 6.5 minutes; pure solvent B ran through the separation column for next 7 minutes. The volume of solvent A was changed back to 100% within next 2 minutes. The flow rate was 2 ml per minute. Pigments were detected at 440 nm. The used protocol of extraction allowed the evaluation of Z, V, A, neoxanthin, lutein and chlorophylls *a* and *b* (Figure 2). The content of xanthophyll cycle pigments was expressed as a deepoxidation state of xanthophyll cycle pigments DEPS = (Z + A)/(Z + A + V).

RESULTS AND DISCUSSION

The response of AO to HL in two lichen species is shown in Table 1. In both species, HL induced an apparent increase in DEPS. The increase is well comparable to the evidence reported for HL-treated higher plants (e.g. Wehner et al. 2006) and lichens (Vráblíková et al. 2006). The amount of total GSH decreased after the HL exposure. The response corresponds to the general trends reported for higher plants (Burritt and Mackenzie 2003).

The aim of the experiments presented in this paper was to test and improve the methods of Z and glutathione quantification in lichens. We may conclude that both used methods are well applicable in the investigation of HL-induced stress in lichens. In Z determination, we used acetone as an extraction medium. Compared to the other options (ethanol, DMSO; see Pfeifhofer

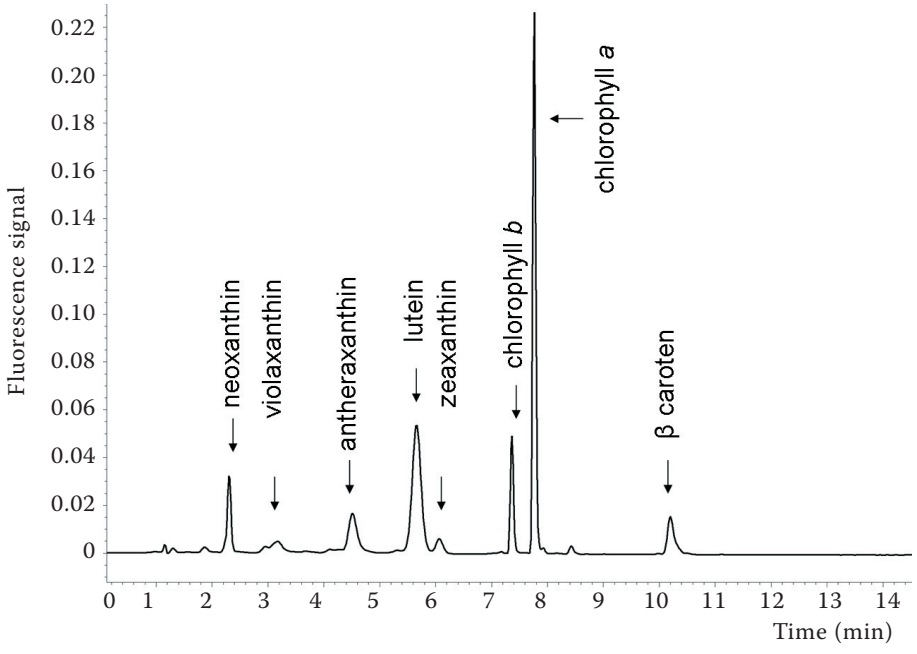

Figure 2. Typical HPLC chromatogram of pigments separation in *L. pustulata*

Table 1. Effect of high light (HL) exposures on deepoxidation state of xanthophyll cycle pigments (DEPS) and total glutathione (GSH) in two lichen species using the methods as described above

	DEPS before HL exposure	DEPS after HL exposure	Total GSH before HL exposure (nmol/g dw)	Total GSH after HL exposure (nmol/g dw)
L. pustulata[1]	0.06938 ± 0.011293	0.544754 ± 0.046581	3354.96837 ± 373.4212	1220.74672 ± 159.7987
U. antarctica[1]	0.138023 ± 0.005171	0.448041 ± 0.072315	1897.08012 ± 232.8332	258.10377 ± 41.26204
U. antarctica[2]	0.232346 ± 0.003363	0.624357 ± 0.052016	1756.85893 ± 800.6829	614.02107 ± 143.5701

The values are means of at least 3 replicates ± standard error; data are taken from the experiments published in [1]Vráblíková et al. (2005), [2]Barták et al. (2004)

et al. 2002), acetone is cheaper and relatively safe. To prevent evaporation of acetone, the extraction and all laboratory manipulations must be done quickly; we recommend to do the extraction on ice crush and to use chilled acetone. DMSO, on the other hand, is a more effective solvent, and the extraction with it requires less labor (Pfeifhofer et al. 2002). Based on our experience, we suggest grinding freeze-dried thalli of foliose lichens for at least 2 minutes in a ball mill. Nevertheless, the used protocol of homogenization cannot be generalized for all lichen species. Thalli of fruticose lichens are more resistant to mechanic destruction; their homogenization would probably require more time and a stronger extraction medium.

REFERENCES

Barták M., Hájek J., Vráblíková H., Dubová J. (2004): High-light stress and photoprotection in *Umbilicaria antarctica* monitored by chlorophyll fluorescence imaging and changes in zeaxanthin and glutathione. Plant Biol., *6:* 333–341.

Bhatacharjee S. (2005): Reactive oxygen species and oxidative burst: Roles in stress, senescence and signal transduction in plants. Curr. Sci., *89:* 1113–1121.

Blokhina O., Virolainen E., Fagerstedt K.V. (2003): Antioxidants, oxidative damage and oxygen deprivation stress: A review. Ann. Bot., *91*: 179–194.

Burritt D.J., Mackenzie S. (2003): Antioxidant metabolism during acclimation of *Begonia* × *erythrophylla* to high light levels. Ann. Bot., *91:* 783–794.

Frank H.A., Cua A., Chynwat V., Young A., Gosztola D., Wasielewski M.R. (1994): Photophysics of the carotenoids with the xanthophylls cycle in photosynthesis. In: Frank H.A. et al. (eds.): The Photochemistry of Carotenoids. Kluwer Academic Publishers, Dordrecht, Netherlands.

Gilmore A.M. (1997): Mechanistic aspects of xanthophyll-cycle dependent photoprotection in higher plant chloroplasts and leaves. Physiol. Plant., *99:* 197–209.

Havaux M., NyiogI K.K. (1999): The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism. Proc. Natl. Acad. Sci. USA, *96:* 8762–8767.

Kranner I. (1998): Determination of glutathione, glutathione disulphide and two related enzymes, glutathione reductase and glucose-6-phosphate dehydrogenase, in fungal and plant cells. In: Varma A. (eds.): Mycorrhizal Manual. Springer Verlag, Berlin, Germany: 227–241.

Kranner I., Birtic S. (2005): A modulating role of antioxidants in desiccation tolerance. Integr. Comp. Biol., *45:* 734–740.

Kranner I., Cram W.J., Zorn M., Wornik S., Yoshimura I., Stabentheiner E., Pfeifhofer H.W. (2005): Antioxidants and photoprotection in a lichen as compared with its isolated symbiotic partners. Proc. Natl. Acad. Sci. USA, *102:* 3141–3146.

Loggini B., Scartazza A., Brugnoli E., Navari-Izzo F. (1999): Antioxidative defense system, pigment composition, and photosynthetic efficiency in two wheat cultivars subjected to drought. Plant Physiol., *119:* 1091–1099.

Noctor G., Foyer C.H. (1998): Ascorbate and glutathione: Keeping active oxygen under control. Annu. Rev. Plant Physiol. Plant Mol. Biol., *49:* 249–279.

Pfeifhofer H.W., Willfurth R., Zorn M., Kranner I. (2002): Analysis of chlorophylls, carotenoids, and tocopherols in lichens. In: Kranner I. et al. (eds.): Protocols in Lichenology. Springer Verlag, Berlin, Germany, 363–378.

Vanacker H., Carver T.L. V., Foyer C.H. (2000): Early H_2O_2 accumulation in mesophyll cells leads to induction of glutathione during the hypersensitive response in the barley-powdery mildew interaction. Plant Physiol., *123:* 1289–1300.

Vráblíková H., Barták M., Wönisch A. (2005): Changes in glutathione and xanthophyll cycle pigments in high light-stressed lichens *Umbilicaria antarctica* and *Lasallia pustulata*. J. Photochem. Photobiol., B – Biol., *79*: 35–41.

Vráblíková H., McEvoy M., Solhaug K.A., Barták M., Gauslaa Y. (2006): Annual variation in photoacclimation and photoprotection of the photobiont in the foliose lichen *Xanthoria parietina*. J. Photochem. Photobiol. B – Biol., *83:* 151–163.

Wehner A., Grasses T., Jahns P. (2006): De-epoxidation of violaxanthin in the minor antenna proteins of photosystem II, LHCB4, LHCB5, and LHCB6. J. Biol. Chem., *281:* 21914–21933.

Weissman L., Garty J., Hochman A. (2005): Rehydration of the lichen *Ramalina lacera* results in production of reactive oxygen species and nitric oxide and a decrease in antioxidants. Appl. Environ. Microbiol., *71:* 2121–2129.

Received on February 26, 2007

Corresponding author:

Mgr. Jana Štepigová, Masarykova Univerzita, Přírodovědecká fakulta, Ústav experimentální biologie, Kotlářská 2, 611 37 Brno, Česká republika
e-mail: pamba@sci.muni.cz