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ORIGINAL PAPER

Cadmium-induced stimulation of stress-protein hsp70 in lichen photobiont *Trebouxia erici*

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Abstract The expression of stress protein 70 (hsp70) was studied in lichen photobiont *Trebouxia erici* during short-term exposition to cadmium and copper (0.0, 1.0, 5.0 and 10.0 μM). We found two isoforms of hsp70 in the untreated as well as in heavy metal-treated cells due to the maintenance of protein homeostasis. Cu-treated cells had a relatively constant amount of hsp70 over all the tested concentrations. However, Cd caused an increase in hsp70 expression, especially at the lowest concentration (1.0 μM). Higher Cd concentrations were associated with acute toxicity and a reduced expression of hsp70 in the cells.

Keywords Hsp70 · Lichens · Photobionts · Stress proteins

Abbreviations
Hsp70 Heat shock protein 70

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Introduction

A lichen's vegetative body (thallus) is composed of a fungal (mycobiont) and a photosynthetic partner (photobiont). However, its sensitivity or tolerance to environmental stresses is determined by the more sensitive biont. Till date, the photobiont has been typically found to be the key element of lichen's sensitivity to environmental pollution (Ahmadjian 1993).

Heavy metal toxicity or tolerance of lichen photobionts in *Trebouxia*, which is the algal partner of more than half of the known lichen species (Ahmadjian 1993), has been experimentally documented (Bačkor et al. 2003, 2004; Pawlik-Skowrońska et al. 2002; Sanità di Toppi et al. 2005). These experiments focused on detoxification mechanisms including cell wall immobilization, free proline synthesis and the activities of some enzymes and thiol compounds.

Extreme environmental conditions trigger changes in the transcript levels of numerous genes encoding proteins thought to have protective functions against stress-related damages. Heat shock proteins (hsp) are a group of highly conserved proteins that are expressed in response to stresses, such as low/high temperature, heavy metal exposure and oxygen depletion (Sanita di Toppi and Gabrielli 1999; Bierkens 2000). In normal cells they are involved in the maintenance of protein homeostasis, i.e. protein folding,

aggregation and trafficking (Hershko 1998; Hightower 1991), and are generally grouped into families according to their molecular weight. Each stress protein family comprises multiple isoforms, each of whose synthesis is independently regulated.

The hsp70 family is one of the most abundant of these proteins. Different organisms produce variable number of hsp70 isoforms (Boorstein et al. 1994). At least, one isoform, heat shock cognate 70 (hsc70), is constitutively expressed and assists in the correct folding of nascent polypeptides (Hartl 1996). The stress-inducible form (hsp70) is considered to have a variety of roles including augmentation of hsc70, refolding partially unfolded proteins and facilitating the removal of denatured proteins (Hartl 1996). Because the concentrations of hsp70 are increased in response to environmental stress, it is used as a biomarker (Bierkens 2000). It has been suggested that hsp70, as a biomarker, integrates overall adverse effects on protein integrity, which are collectively summarized by the term 'proteotoxicity' (Sanders 1993). Measurements of hsp70 can provide evidence that organisms have been exposed to toxicants. However, to our knowledge, hsp70 proteins have never been studied in lichens or in their symbionts.

Western blotting, slot blotting, radioimmunoassays (RIAs) and enzyme-linked immunosorbent assay (ELISAs) are used to detect and quantify stress proteins (Currie and Tufts 1997; Werner and Nagel 1997). These methods rely on the availability of cross-reactive antibodies for the proteins under investigation. The highly conserved nature of heat shock proteins signifies that many antibodies are species cross-reactive. Western blotting is the most widely applied technique for assaying stress proteins. It is a method in molecular biology to detect protein in a complex mixture of biomolecules using specific probes, such as an antibody or streptavidin specific to a protein or a protein tag, such as the hexa-histidine, biotin, c-myc or FLAG tag.

The aim of this work was to observe expression of hsp70 in axenic cultures of lichen photobiont *Trebouxia erici* and compare its levels after short-term (24-h) exposure to copper and cadmium.

Material and methods

Photobiont

Wild-type lichen photobiont *Trebouxia erici* Ahmadjian (UTEX 911) was used in this study. The semisolid medium used in this study was based on Bold's Basal Medium (BBM 3N) containing 10 g l^{-1} casein hydrolysate, 20 g l^{-1} glucose and 15 g l^{-1} agar with the pH maintained at 6.5 (Ahmadjian 1993). The cultures were maintained at 22°C under a 16-h photoperiod (30 μmol m^{-2} s^{-1} PPFD from cool white fluorescent lights).

Copper and cadmium stresses

Three-week-old photobiont cultures in exponential growth phase were gently separated from the surface of the media, and to disrupt the cell aggregates, the cell mass was passed through a phytoplankton net with a pore size 50 μm. The cells were immediately suspended in 5 mM HEPES buffer at pH 6.5 producing an initial culture density of approximately 80 mg DW l^{-1}. Photobionts were treated with 50 ml of Cu and Cd (0.0, 1.0, 5.0 and 10.0 μM), and placed in a climatic chamber under the same conditions as above for 24 h. Cd was supplied as $Cd(NO_3)_2$ and copper as $CuCl_2 \cdot 2H_2O$.

Intracellular Cu and Cd accumulation

Photobiont cells, treated for 24 h with Cu and Cd (0.0–10.0 μM) in 5 mM HEPES buffer at pH 6.5, were centrifuged and subsequently resuspended in 10 ml of 2.5 mM Na_2-EDTA (analytical grade) for 15 min to remove superficially bound metal. Photobiont cells washed with Na_2-EDTA were collected by centrifugation, and the pellets were rinsed with 10 ml of deionised water. Water-rinsed photobiont cells were mineralized with a mixture of 65% HNO_3 and 30% H_2O_2 (2:1, v/v). Metal concentrations in cells were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES). Three replicates were used for each treatment.

Protein content

Photobiont cells, treated for 24-h with Cu and Cd (0.0–10.0 μM) in 5 mM HEPES buffer at pH 6.5, were centrifuged at 15,000 × *g*, and the pellets were ground and homogenized in an ice-cold mortar in phosphate buffer (50 mM, pH 6.5). After centrifugation at 15,000 × *g* at 4°C for 20 min, water-soluble proteins content from supernatants were measured according to Bradford (1976) by the use of the Bio-Rad protein assay (Bio-Rad Laboratories Inc., Hercules, California) and bovine serum albumin as a calibration standard.

Western blot analysis

The cells were scraped and lysed in Laemmli sample buffer (100 mM Tris [pH 6.8], 2% sodium dodecyl sulfate [SDS] and 10% glycerol). The extracts of total proteins were assayed with a DC protein assay kit (Bio-Rad Laboratories Inc., Hercules, California), and equal amounts (20 μg) were separated on 12% SDS-polyacrylamide gel. Each protein sample was electroblotted onto Immobilon-P transfer membrane (Millipore, Bedford, MA) in a buffer containing 192 mM glycine, 25 mM Tris and 10% methanol. The membranes were blocked for 1 h in 5% powdered non-fat milk in wash buffer (0.5% Tween-20 in 20 mM Tris [pH 7.6] and 140 mM NaCl) and incubated overnight at 4°C with primary antibodies (mouse monoclonal IgG1 anti-HSP70, 1:5000, MA3-006, Affinity BioReagents, Golden, Colorado). After the washing of membranes, secondary horseradish peroxidase-conjugated antibodies (anti-Mouse IgG F (AB′) 2, 1:10000, PI-31461, Pierce, Rockford, Illinois) were added for 1 h. The membranes were washed, and antibody reactivity was visualized with ECL Western blotting substrate (PI-32106) reagent (Pierce) using Kodak Biomax film (#1788207, Sigma-Aldrich Cor., St. Louis, Missouri). Equal sample loading was verified by non-specific amidoblack staining of membranes after immunoblotting.

Statistical analysis

One-way analysis of variance and Tukey's test (MINITAB Release 11, 1996, State College, Pennsylvania, USA) were used to assess the statistical ($P \leq 0.05$) mean differences among quantitative parameters (metal uptake and protein content).

Results and discussion

Short-term exposure (24-h) to heavy metals revealed dose-dependent accumulation of both the metals tested (Fig. 1). In general, high exogenous metal concentrations caused an increase in intracellular uptake of both Cu and Cd. Cu at 5.0 and 10.0 μM resulted in a significant decline in protein content of *T. erici* cells (Fig. 1). However, Cd did not have any significant effect on protein content.

In the present study, the expression of hsp70 was detected in the cells of lichen photobiont *T. erici* (Fig. 2). Two isoforms represented hsp70, and were detectable in controls as well as metal-treated cells. Cu at all the concentrations (1.0–10.0 μM) did not affect the expression of hsp70. However, Cd could greatly stimulate the expression of hsp70. The highest expression of hsp70 was induced by 1.0 μM Cd, and no further increase in its expression occurred with higher Cd concentrations (5.0 and 10.0 μM) (Fig. 2).

Although the uptake of both the metals in *T. erici* was in general comparable over selected concentrations, we reported earlier differences in biological responses when both these metals were compared together (Bačkor et al. 2006). Cu has been reported to be a redox-reactive metal (Sanità di Toppi and Gabrielli 1999), and is capable of increasing the levels of membrane lipid peroxidation (TBARS), decreasing chlorophyll *a* fluorescence and oxygen evolution rate and altering the composition of assimilation pigments (Bačkor et al. 2003, 2004). Cd, on the other hand, caused a decrease in chlorophyll *a* fluorescence and oxygen evolution rate; however, the composition of assimilation pigments and the levels of membrane lipid peroxidation were stable over the concentrations tested (Bačkor et al. 2006).

In lichens, it has been demonstrated that there is a decline in copper protein content over the course of experiment (Monnet et al. 2006), similar to what we observed in the axenic cultures

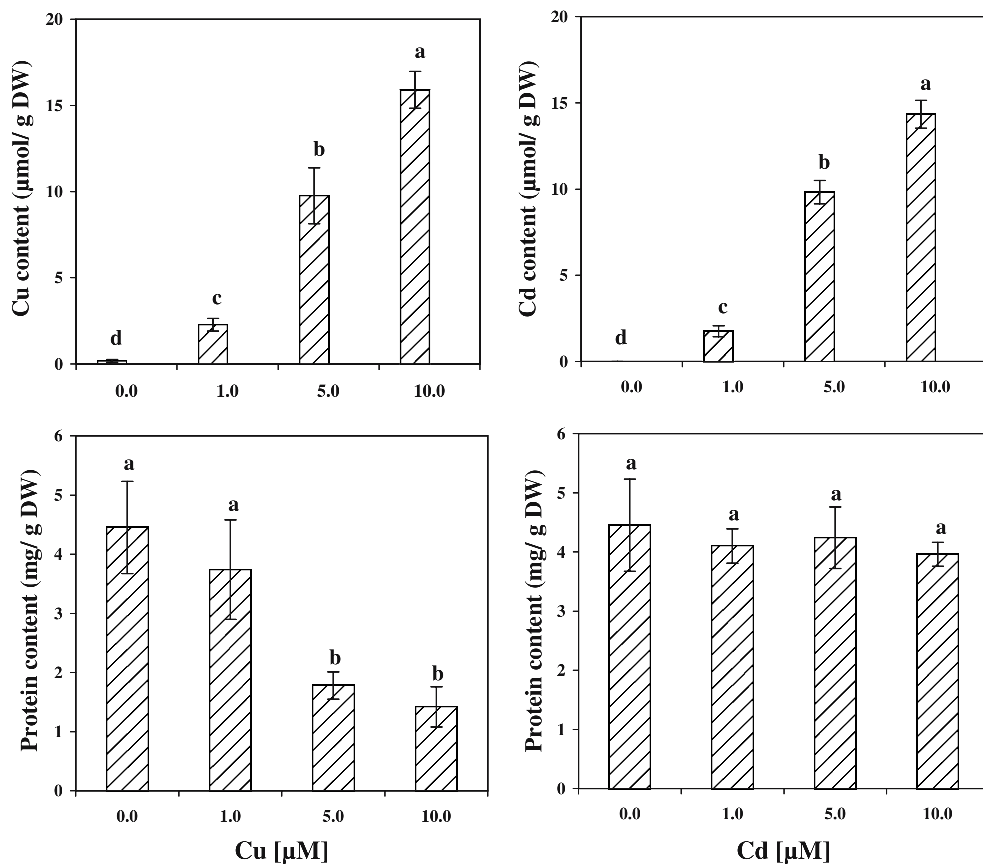
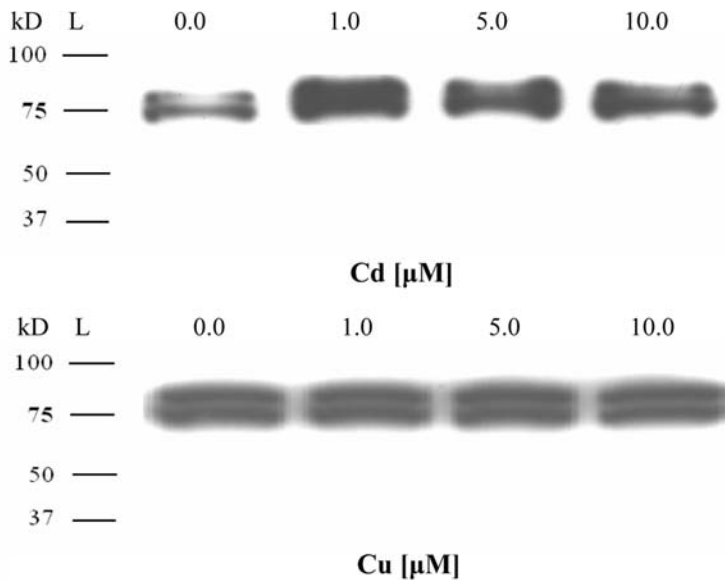


Fig. 1 Effects of different concentrations of exogenous copper (Cu) and cadmium (Cd) on their intracellular uptake and protein content by *Trebouxia erici* photobiont following 24-h exposure. Means with common letters are not significantly different at $P \leq 0.05$, according to Tukey's test

Fig. 2 Western blot analysis of stress-protein hsp70 in sample extracts of *Trebouxia erici* photobiont exposed to different concentrations of copper (Cu) and cadmium (Cd) for 24-h. L = ladder



of lichen algal partner. Although there is a lack of information on water-soluble protein content in lichen algae, the protein content of untreated cells of *T. erici*, in the present study, was comparable to the results obtained in *Chlorella* spp. by using mechanical disruption of the cells and Bradford assay (Meijer and Wiffels 1998).

When organisms are subjected to treatments causing protein damage (proteotoxicity), some of the constitutively expressed stress-proteins are up-regulated proportionally to the degree of stress (Bierkens 2000). Under these adverse conditions stress-proteins are thought to counter proteotoxic effects by (i) preventing the protein denaturation and holding them in the state of folding of assembly to facilitate repairs, and (ii) promoting the degradation of abnormal proteins. However, organisms respond to different levels of stress by activating different stress proteins. We confirmed in our study that a family of hsp70 can be a very good biomarker for assessment of metal toxicity in lichen algae *T. erici*.

Each stress-protein family is comprised of isoforms, each of whose synthesis is independently regulated. In *T. erici* photobiont, hsp70 was represented by two isoforms. The antibody probably recognized both hsc70 and hsp70 isoforms, similar to what was reported by Ireland et al. (2004). We also found detectable levels of both the isoforms of hsp70 in metal-untreated cells. In normal cells, the stress proteins are involved in the maintenance of protein homeostasis, e.g. protein folding, aggregation and trafficking (chaperoning). It is known that cultivation medium always contains (nutritional levels of) some essential heavy metals. Since expression of stress proteins in the cells is very sensitive to the presence of metals, detectable levels of stress proteins is due to the presence of heavy metals in cultivation medium.

As organisms respond to different levels of stress by employing the activities of different stress-proteins (Bierkens 2000), screening for one particular class of stress-proteins may not provide a sensitive marker for a wide range of the pollutants. However, as noted previously, families of stress-proteins, such as hsp70 and hsp60, are the two most prominent and frequently referred to in the context of heavy metal toxicity and tolerance. Bierkens et al. (1998) found that in green alga *Raphidocelis subcapitata* hsp70 was induced in a dose-dependent way in response to different chemicals including the metals, such as Zn and Se. The expression of hsp70 was found at concentrations bellow the range of classical cytotoxicity testing (e.g. growth inhibition).

We also observed a differential response of lichen algae *T. erici* to Cu and Cd. Redox-reactive metal Cu did not increase the expression of hsp70. It has been reported earlier that endemic sponges from lake Baikal respond to increased Pb and Zn, but not to Cu, by strong induction of hsp70 expression (Efremova et al. 2002). However, Cu has been found to be a strong activator of hsp60 synthesis in the halophyte *Mesembryanthemum crystallinum* (Thomas et al. 1998). In the present study, Cd was selected as the representative of a non-essential toxic heavy metal, and it caused a strong induction of hsp70 synthesis in *T. erici* photobiont cultures. Cd at a lower concentration (1.0 μM) caused the strongest induction of hsp70 synthesis with its increasing concentrations resulting in a decline in hsp70 expression. This phenomenon is not surprising and has been observed previously in the marine macroalgae *Fucus serratus* and aquatic plant *Lemna minor* in response to Cd stress (Ireland et al. 2004). The levels of stress-proteins probably cannot continuously increase because the cost of hsp expression will outweigh its benefits (Pyza et al. 1997). Another reason for a decrease in hsp expression in Cd-treated photobiont cells, as observed in the present study, may perhaps be because Cd at higher concentrations inhibits some aspects of metabolisms including photosynthesis and protein synthesis (Bačkor unpublished results).

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