

## Lichen Substances Affect Metal Adsorption in *Hypogymnia physodes*

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**Abstract** Lichen substances are known to function as chelators of cations. We tested the hypothesis that lichen substances can control the uptake of toxic metals by adsorbing metal ions at cation exchange sites on cell walls. If true, this hypothesis would help to provide a mechanistic explanation for results of a recent study showing increased production of physodalic acid by thalli of the lichen *Hypogymnia physodes* transplanted to sites with heavy metal pollution. We treated cellulose filters known to mimic the cation exchange abilities of lichen thalli with four lichen substances produced by *H. physodes* (physodic acid, physodalic acid, protocetraric acid, and atranorin). Treated filters were exposed to solutions containing seven cations ( $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Na}^+$ ), and changes to the solution concentrations were measured. Physodalic acid was most effective at influencing metal adsorption, as it increased the adsorption of  $\text{Fe}^{3+}$ , but reduced the adsorption of  $\text{Cu}^{2+}$ ,  $\text{Mn}^{2+}$ , and  $\text{Na}^+$ , and to a lesser extent, that of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Reduced  $\text{Na}^+$  adsorption matches with the known tolerance of this species to NaCl. The results may indicate a possible general role of lichen substances in metal homeostasis and pollution tolerance.

**Keywords** Depsides · Depsidones · Pollution tolerance · Epiphytic lichens · Transition metals · Alkaline earth metals · Alkali metals

### Introduction

Lichens are notable for the production of unique lichen substances, including more than 800 aliphatic, cycloaliphatic, aromatic, and terpenic compounds (Huneck and Yoshimura, 1996). Lichens are also sensitive to a variety of air pollutants and are widely used as

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biomonitors of air quality. Recently, Białońska and Dayan (2005) linked lichen secondary chemistry with the use of lichens as biomonitors in a study of the common lichen *Hypogymnia physodes*. This lichen has often been employed as a model species in ecophysiological and biomonitoring studies because of its abundance and its moderate sensitivity to SO<sub>2</sub> and heavy metals. In Europe, *H. physodes* has declined in many areas with significant pollutant load. Białońska and Dayan (2005) found that thalli of *H. physodes* transplanted to areas polluted with heavy metals and inorganic S compounds exhibited significantly lower concentrations of atranorin, physodic acid, and 3-hydroxyphysodic acid and significantly higher concentrations of physodalic acid. They postulated that physodalic acid might participate somehow in the stress response of *H. physodes* to toxic effects of heavy metals and acidic inorganic S compounds. This was the first time that the content of lichen substances had been linked to pollution tolerance. However, the authors did not provide any mechanistic hypothesis to explain any such relationship.

Our hypothesis is that the presence of lichen compounds significantly reduces toxic effects of heavy metals to living cells by controlling their extracellular adsorption and thereby influencing their intracellular uptake. Specifically, we tested the hypothesis that physodalic acid affects the adsorption of metal ions at cation exchange sites and thereby contributes to the heavy metal tolerance of *H. physodes*. To test our hypothesis, we used cellulose filters coated with physodalic acid and other lichen substances of *H. physodes* (atranorin, physodic acid, and protocetraric acid) as a standardized model system containing numerous hydroxyl groups as cation exchange sites (Hauck et al., 2007). Lichen cell walls and exopolysaccharide matrices harbor many hydroxylic and carboxylic binding sites for cations, from which delayed intracellular uptake is possible (Hauck et al., 2006). Metal ions chosen for the study included the transition metals Cu<sup>2+</sup>, Fe<sup>2+</sup>, Fe<sup>3+</sup>, and Mn<sup>2+</sup> and the alkaline earth metals Ca<sup>2+</sup> and Mg<sup>2+</sup>, which (1) occurred in measurable concentrations in the stemflow of trees with *H. physodes* in ecosystem studies conducted in Europe and North America (Hauck, 2003); (2) are essential macro- or micronutrients; and (3) are, in the case of the transition metals, toxic to *H. physodes* when concentrations significantly exceed the amount required for nutrition (Hauck, 2003). The alkali metal Na<sup>+</sup> was studied in addition, as it is often the most common metal in precipitation.

If our hypothesis is correct, the presence of lichen compounds on cellulose filters will significantly alter the adsorption of metal cations from solution. We were especially interested in the effects of physodalic acid on cation uptake inasmuch as this compound was specifically mentioned by Białońska and Dayan (2005) to be involved in the stress response of *H. physodes*.

## Methods and Materials

*Hypogymnia physodes* (L.) Nyl. is a common foliose lichen of the temperate and boreal zones of the northern hemisphere, which primarily occurs as an epiphyte (McCune, 2002). Seven lichen substances have been identified in *H. physodes* (McCune, 2002), which are either located in the cortex (atranorin, chloroatranorin) or in the medulla (physodic, 3-hydroxyphysodic, physodalic, protocetraric, 2'-*O*-methylphysodic acids). The two latter substances are found only in minor amounts (McCune, 2002). The cortical lichen substances of *H. physodes* are depsides, whereas the medullary ones belong to the depsidones (Huneck and Yoshimura, 1996).

Pure atranorin, physodic acid, physodalic acid, and protocetraric acid from the collection of S. Huneck were dissolved in acetone (2.5 mM) and applied to ash-free cellulose filter

**Table 1** Cation adsorption from micromolar salt solutions by filter paper soaked with 2.5 mM of different lichen substances

	Cu <sup>2+</sup>	Fe <sup>2+</sup>	Fe <sup>3+</sup>	Mn <sup>2+</sup>	Ca <sup>2+</sup>	Mg <sup>2+</sup>	Na <sup>+</sup>
Incubation medium	8	85	85	35	260	110	800
Physodalic acid	1.21±0.27 (119%) <sup>a</sup> <sup>b</sup>	65.6±2.6 (-3%)	1.2±1.1 (-95%) <sup>b</sup>	23.7±0.4 (23%) <sup>b</sup>	242±3 (4%) <sup>b</sup>	90.9±2.2 (5%) <sup>b</sup>	837±35 (48%) <sup>b</sup>
Physodic acid	0.71±0.16 (28%)	68.6±2.2 (1%)	14.0±3.1 (-44%) <sup>b</sup>	19.1±0.2 (-1%)	238±2 (3%)	84.2±1.3 (-3%)	759±31 (34%) <sup>b</sup>
Protocetraric acid	1.02±0.12 (84%)	69.8±1.4 (3%)	14.4±2.1 (-43%) <sup>b</sup>	22.8±0.5 (18%) <sup>b</sup>	238±3 (2%)	89.6±2.1 (3%)	740±37 (31%) <sup>b</sup>
Atranorin	0.76±0.15 (37%)	63.7±2.1 (-6%)	27.3±3.6 (8%)	20.3±0.4 (9%)	245±5 (5%) <sup>b</sup>	88.7±1.9 (2%)	782±26 (38%) <sup>b</sup>
Control	0.55±0.12	67.7±1.2	25.2±3.1	19.3±0.3	233±1	86.8±2.5	565±64

Micromolar salt solutions are chloride salts, except for Na<sub>2</sub>SO<sub>3</sub>. All concentrations in μM. Arithmetic means ± standard error, *N*=5.

<sup>a</sup> Relative change of metal ion concentration after shaking two filter strips impregnated with different lichen substances or not (control) for 1 hr in salt solution. Control is set to 100%. Positive values indicate higher and negative values indicate lower metal concentration in the solution.

<sup>b</sup> Significant difference from control (Duncan's multiple range test, *P*≤0.05, *df*=20).

paper (Blue Ribbon Filters, Schleicher & Schuell, Dassel, Germany) by shaking filter strips (ca. 20 cm<sup>2</sup>, 160 mg) in the lichen substance solution for 1 hr. Cellulose filters consist of randomly interlaced fibers that are littered with exchange sites that bind to metal ions, similar to the cell wall surfaces of lichens (Hauck et al., 2007). Untreated filter strips were used as controls. Two strips of impregnated filters per replicate were exposed for 1 hr to 25 ml of micromolar solutions of CuCl<sub>2</sub>, FeCl<sub>2</sub>, FeCl<sub>3</sub>, MnCl<sub>2</sub>, CaCl<sub>2</sub>, MgCl<sub>2</sub>, or Na<sub>2</sub>SO<sub>3</sub>; the concentrations applied (Table 1) referred to the optimum measuring range of the AAS (Vario 6, Analytik Jena, Jena, Germany; Cu, Fe, Mn, Ca, Mg) or inductively coupled plasma-atomic emission spectrometry (Spectraflame, Spectro Analytical Instruments, Fitchburg, MA, USA; Na) used for chemical analysis. After incubation, the filter paper was removed with forceps and metal concentrations in the solution were analyzed. The experiment was run in five replicates. Statistical analyses were conducted with SAS 6.04 software (SAS Institute, Cary, NC, USA). Duncan's multiple range test was applied to test for significance of differences between means after testing for normal distribution with the Shapiro–Wilk test.

## Results

For all but one of the cations studied, the presence of lichen compounds on filter strips resulted in significantly reduced removal of the cations from solution, indicating that lichen compounds prevent the binding of cations to exchange sites on the filters. Physodalic acid was especially inhibitory inasmuch as filters coated with this lichen compound adsorbed from solution significantly less Cu<sup>2+</sup>, Mn<sup>2+</sup>, Na<sup>+</sup>, Ca<sup>2+</sup>, and Mg<sup>2+</sup> than control samples of filters not soaked with a lichen substance (Table 1). Adsorption of Mn<sup>2+</sup> was also reduced by protocetraric acid, as was that of Ca<sup>2+</sup> by atranorin. All studied lichen substances also inhibited the adsorption of Na<sup>+</sup>. Lichen compounds caused significantly enhanced adsorption of only one metal cation, and this was Fe<sup>3+</sup>. While the concentrations of Fe<sup>2+</sup>

were not affected by any of the lichen substances,  $\text{Fe}^{3+}$  was nearly completely removed from solution by filters coated with physodalic acid. Physodic and protocetraric acids significantly increased removal of  $\text{Fe}^{3+}$  from the incubation medium by 43–44% (Table 1).

## Discussion

Our results provide a mechanistic explanation for the hypothesis of Białońska and Dayan (2005) that enhanced synthesis of physodalic acid in polluted areas is a stress response of *H. physodes* to heavy metal pollution. High ambient concentrations of both  $\text{Cu}^{2+}$  and  $\text{Mn}^{2+}$  have been shown to limit the abundance of *H. physodes* in the field (Hauck, 2003; Hauck and Paul, 2005). An inhibition of the adsorption of these ions in the apoplast would, therefore, increase the tolerance of *H. physodes* to  $\text{Cu}^{2+}$  and  $\text{Mn}^{2+}$ .

The strong reduction of the  $\text{Fe}^{3+}$  concentration in the incubation medium by physodalic acid suggests that this lichen substance forms complexes with  $\text{Fe}^{3+}$ . Evidence for the existence of such complexes with  $\text{Fe}^{3+}$  has been found for several lichen substances (Engstrom et al., 1980; Hauck et al., 2007) belonging, like physodic acid, to the depsidones (fumarprotocetraric and norstictic acids), the depsides (divaricatic acid), the pulvinic acid derivatives (pulvinic and rhizocarpic acids), or the anthraquinones (parietin). Complex formation with transition metals has also been established for other combinations of lichen substances and metal ions (e.g.,  $\text{Cu}^{2+}$ ) and was mostly discussed in the light of immobilization of potentially toxic ions (Takani et al., 2002).

Occurrence of  $\text{Fe}^{3+}$ -adsorbing lichen substances in lichens of sites with low Fe availability, but lack in most lichen species of Fe-rich substrate, suggests that  $\text{Fe}^{3+}$  bound in complexes with lichen substances is subsequently taken up into the cytoplasm (Hauck et al., 2007). The only  $\text{Fe}^{3+}$  adsorbing lichen substances known, so far, from lichen species of Fe-rich substrate, viz. norstictic and rhizocarpic acids, simultaneously reduce the adsorption of  $\text{Fe}^{2+}$  (Hauck et al., 2007). Increased uptake of  $\text{Fe}^{3+}$  in *H. physodes* by physodalic acid and other lichen substances would increase its tolerance to  $\text{Mn}^{2+}$ , as Mn-related damage depends on the Mn/Fe ratio (Hauck and Paul, 2005).

If our hypothesis is correct, the increased production of physodalic acid by *H. physodes* in polluted areas observed by Białońska and Dayan (2005) is a response to pollution stress. However, the reduced concentration of other lichen substances (atranorin, physodic acid, and 3-hydroxyphysodic acid) also requires explanation. The decline of physodic acid in thalli of *H. physodes* transplanted to sites with heavy metal pollution could be due to conversion into the chemically related physodalic acid. Protocetraric acid, which is even more closely related to physodalic acid than physodic acid (Huneck and Yoshimura, 1996), was not included in the analyses of Białońska and Dayan (2005) and should be investigated for possible stress responses. Inhibition of  $\text{Na}^+$  adsorption by all investigated lichen substances (Table 1) agrees with the known tolerance of *H. physodes* to NaCl (Punz, 1979).

The putative ability of physodalic acid to increase heavy metal tolerance would explain why *H. physodes* was less subject to pollutant-caused declines in the past than other European *Hypogymnia* species, including *Hypogymnia farinacea*, *Hypogymnia tubulosa*, and *Hypogymnia vittata*, as *H. physodes* is the only European species with physodalic acid (McCune, 2002). The assumed protective effect of physodalic acid in *H. physodes* is probably limited because this substance is primarily produced by the fungal hyphae of the medulla and the algal layer (McCune, 2002), while the cortical atranorin did not affect the adsorption of transition metals (Table 1). The absence of a protective lichen substance from the cortex would explain the moderate heavy metal tolerance of *H. physodes* (Hauck, 2003;

Hauck and Paul, 2005). *Lecanora conizaeoides*, a markedly Mn-tolerant lichen (Hauck and Paul, 2005), contains the depsidone fumarprotocetraric acid in the entire thallus. This substance also reduces  $Mn^{2+}$  adsorption and is even more effective at adsorbing  $Fe^{3+}$  than physodalic acid (Hauck et al., 2007). Though our experiment suggests a protective role of physodalic acid, it has to be kept in mind that adsorption rates on fungal and algal cell walls might differ from that of cellulose fibers. Moreover, other mechanisms including immobilization in polyphosphate bodies and phytochelatinates are involved in the heavy metal tolerance of lichens (Hauck and Paul, 2005).

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