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Cadmium induces a novel metallothionein and phytochelatin 2 in an aquatic fungus ☆

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Abstract

Cadmium stress response was measured at the thiol peptide level in an aquatic hyphomycete (*Heliscus lugdunensis*). In liquid culture, 0.1 mM cadmium increased the glutathione (GSH) content and induced the synthesis of additional thiol peptides. HPLC, electrospray ionization mass spectrometry, and Edman degradation confirmed that a novel small metallothionein as well as phytochelatin (PC2) were synthesized. The metallothionein has a high homology to family 8 metallothioneins (http://www.expasy.ch/cgi-bin/lists?metallo.txt). The bonding of at least two cadmium ions to the metallothionein was demonstrated by mass spectrometry (MALDI MS). This is the first time that simultaneous induction of metallothionein and phytochelatin accompanied by an increase in GSH level has been shown in a fungus under cadmium stress, indicating a potential function of these complexing agents for in vivo heavy metal detoxification. The method presented here should be applicable as biomarker tool.

Keywords: Cadmium; Phytochelatin; Metallothionein; Mass spectrometry; ESI; MALDI-PSD; Fungi; Aquatic hyphomycetes; *Heliscus lugdunensis*

Heavy metals exert a distinct selective pressure on those organisms that have developed tolerance/resistance mechanisms to withstand high concentrations of such pollutants, though impoverished fungal communities in aquatic habitats persist when exposed to heavy metal pollution [1]. The question is which strategies allow them to survive in such harsh environments.

In fungal cells, a sophisticated network exists to buffer toxic concentrations of heavy metals in their environment and to regulate intracellular concentrations [1,2].

Metallothioneins are gene-encoded, cysteine-rich metal-binding proteins of low molecular weight. They have been found in fungi and other kingdoms of life [3]. MTs bind heavy metals through clusters of thiolate bonds. Based on sequence similarities and phylogenetic relationships, they have been subdivided into several families [4] (http://www.expasy.ch/cgi-bin/lists?metallo.txt). Their physiological functions have not yet been fully elucidated.

Brewer's yeast *Saccharomyces cerevisiae* contains a multigene MT (*CUP 1*) family, which plays the dominant role for Cu detoxification [5,6]. Some open reading frames coding for putative MTs were identified on the

☆ *Abbreviations:* PC, phytochelatin; MT, metallothionein; GSH, glutathione; ESI, electrospray ionization; MALDI-PSD-MS, matrix-assisted laser desorption/ionization post-source decay mass spectrometry; RP-HPLC, reversed phase-high performance liquid chromatography; SEC, size-exclusion chromatography.

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chromosomes of different fungi, such as the ascomycete *Podospora anserina* [7], the discomycete *Pyrenopeziza brassicae* [8], the arbuscular mycorrhizal fungus *Gigaspora margarita* [9], and the ectomycorrhizal ascomycete *Tuber borchii* [10].

Obviously, fungal MTs can be implicated in a multitude of physiological conditions, e.g., changing environmental conditions (for example, high heavy metal content), developmental processes, and redox status [3,8,11].

In fungi, MTs are predominantly induced by Cu. Only upon Cu exposure, the ascomycete *Neurospora crassa* synthesizes a MT [12–14] which belongs to family 8 of the MT classes (http://www.expasy.ch/cgi-bin/lists?metallo.txt). Small copper-binding cysteine rich (30%) MTs were isolated from the ectomycorrhizal fungi *Laccaria laccata* and *Paxillus involutus*, and characterized on the basis of their molecular masses and spectroscopic features [15]. The induction of fungal MTs by Cd has been reported for *Beauveria bassiana* and a Cd-resistant strain of *S. cerevisiae* [16,17]. In addition to Cd, Cu induced the expression of a MT gene in *Colletotrichum gloeosporioides* [18]. *Candida glabrata* produces a MT in response to high concentrations of Cu, but synthesizes mainly phytochelatins in response to Cd stress [19,20].

Phytochelatins were first described in Cd exposed *Schizosaccharomyces pombe* and named cadystins [21]. Shortly thereafter, peptides of the same structure were described from plant cells [22]. PCs are the most common name for such glutathione-related metal-complexing peptides with the general structure (γ-GluCys)$_n$-Gly) and occur in fungi and plants [23,24]. Unlike MTs, PCs are products of a biosynthetic pathway. The resulting peptides of the phytochelatin synthase action are of varying chain lengths. In the yeast *S. pombe*, the metal binding by PCs is the main Cd detoxification mechanism [2]. In *C. glabrata*, glutathione and PC-capped CdS crystallites occur [25]. In *S. cerevisiae* and *N. crassa*, only γ-(EC)$_2$Gly (PC2) was shown [26]. In *S. pombe* [21] and the zygomycete *Mucor racemosus* [27], Cd-induced PC2 and PC3 were identified. Possibly, *Schizosaccharomyces octosporus* synthesizes higher molecular PCs (PC2–6) under Cd stress, but these peptides were not unequivocally identified [28].

The use of MTs and PCs as biomarker tools in fungi would be more efficient if the analytical protocols allowed us to differentiate and evaluate the substances and their heavy metal-chelating properties. Here we describe the simultaneous determination of Cd-induced peptides and their characterization by different sequencing techniques in the aquatic hyphomycete *Heliscus lugdunensis*.

Materials and methods

Organism and site of its isolation. A single conidium of *H. lugdunensis* Sacc. et Thérry strain H8-2-1 was isolated from site H8 in the Mansfelder Land according to Krauss et al. [29]. The stream is moderately polluted by heavy metals (0.05 μM Cd, 0.3 μM Cu, 15 μM Zn, and 7 μM Mn among other cations and anions [29].

Media and growth conditions. The fungus was maintained on malt extract agar (1.0% malt extract, 1.5% agar) according to Krauss et al. [29]. To investigate the effect of heavy metals, the fungi were grown in liquid medium (0.5% malt extract, Merck; 0.1% peptone, Difco). Fifteen agar plugs (Ø 7 mm) overgrown with fungal mycelium were homogenized in 15 mL liquid medium. One milliliter of mycel homogenate was used to inoculate 75 mL liquid medium in 100 mL Erlenmeyer flasks. After 4 days of cultivation at 14 °C and 120 rpm in the dark, the mycelium was homogenized under sterile conditions. The main culture was started by inoculation with the homogenized mycelium corresponding to 7 μg dry weight. After 3 days of cultivation at 14 °C and 120 rpm in the dark, sterile-filtered 0.1 mM $CdCl_2$ solution was added and the culture was incubated for 3 further days. Mycelium from a 6-day-old culture without Cd was used as a control.

Extraction and HPLC. Freshly harvested mycelium (Whatman filter 3) was washed twice with distilled water, tamped dry between filter paper, and stored at −80 °C for 1 day. The frozen mycelium was ground with mortar and pestle in liquid nitrogen and extracted with 1 N NaOH (containing 1 mg $NaBH_4$ mL^{-1}) in a ratio of 1:2. After 15 min at 20 °C, samples were centrifuged at 11,000*g*. The supernatant was acidified with 70 μL of 3.6 N HCl per 250 μL sample to lower the pH to 1.5. After 15 min on ice and centrifugation for 5 min at 11,000*g*, the supernatant was immediately transferred for analysis. MT and PC were determined by RP-HPLC using an octadecyl reversed phase column (SuperPac Pep-S, Pharmacia, 5 μm) and a linear gradient of 2–20% acetonitrile (ACN) in water (adjusted with trifluoroacetic acid to pH 3.0) for 20 min, followed by 5 min of 20% ACN. The flow rate was 1 mL min^{-1}. The thiol-specific detection was realized by online-post-column derivatization with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (0.3 mM DTNB in 50 mM KH_2PO_4, pH 8.0, Ellman reagent) at a flow rate of 0.4 mL min^{-1}. The thiolic compounds were detected at 410 nm.

In order to exclude the detection of other non-thiolic compounds absorbing at 410 nm, the post-column derivatization was repeated with KH_2PO_4 without Ellman reagent as a negative control [30].

For MALDI-MS analysis of the Cd complexes, no acidified extracts of the mycelium were centrifuged at 11,000*g*. The supernatant was fractionated according to molecular mass distribution by size-exclusion chromatography (SEC) using a Superdex Peptide HR 10/30 column with an exclusion range of 100–7000 Da (Pharmacia Biotech). Cd-induced substances were eluted isocratically with 50 mM $(NH_4)HCO_3$, pH 7.9, and a flow rate of 0.8 mL min^{-1}. The detection wavelengths were set to 215 and 254 nm, the latter being the characteristic absorption wavelength of the cadmium sulfide bond.

Mass spectrometry and Edman degradation. HPLC fractions were analyzed by ESI-MS and ESI-MS/MS with an ion trap mass spectrometer (ESQUIRE-LC, Bruker Daltonik, Bremen, Germany). The samples were injected with a syringe pump at a flow rate of 2 μL min^{-1}. For MS/MS experiments, ions were isolated in a mass window of 4 Da. MALDI-PSD mass spectra were acquired on a reflectron TOF mass spectrometer (REFLEX II, Bruker Daltonik, Bremen, Germany). The acceleration voltage was 20 kV and the reflectron voltage was 22.5 kV. The reflectron voltage was reduced in 14 steps to obtain MALDI-PSD spectra. The samples were prepared according to the dried droplet method with a saturated solution of α-cyano-4-hydroxycinnamic acid matrix dissolved in 0.1% trifluoroacetic acid/acetonitrile (2:1).

The peptide II from RP-HPLC (Fig. 1B) was subjected to N-terminal amino acid sequence analysis by Edman degradation. Fifteen and twenty steps, respectively, were sequenced on a 476A protein sequencer (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions. Since the Cys residues were unmodified and gave no PTH-signals in the respective cycles, cysteins and the missing C-terminal residues were confirmed by MS-sequencing.

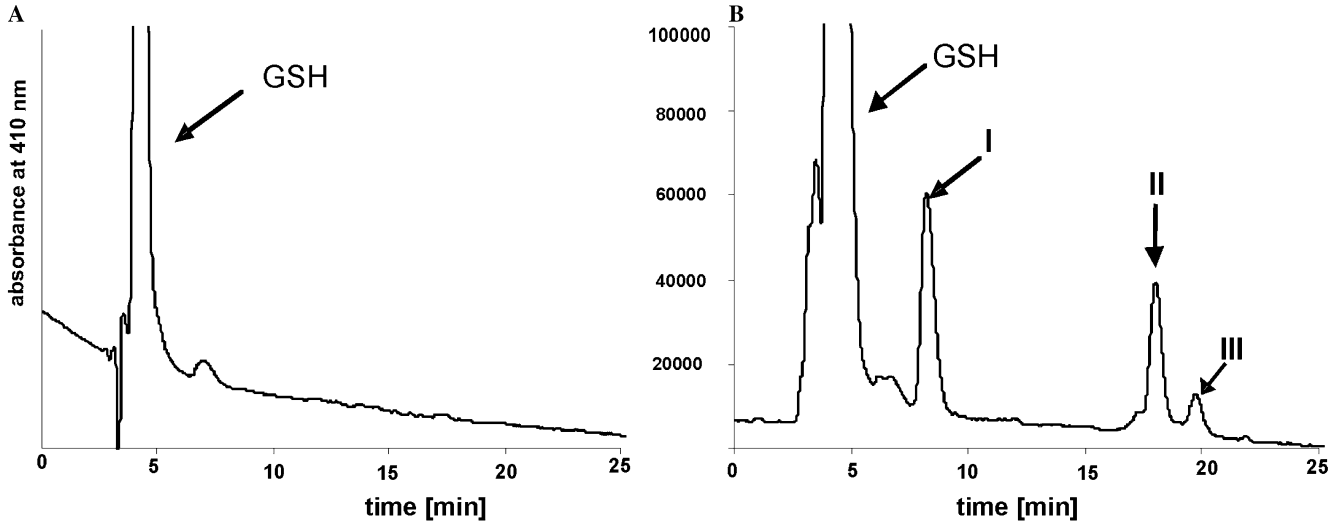

Fig. 1. Reversed phase-HPLC and post-column Ellman derivatization of extracts of *H. lugdunensis* (A) control; (B) exposed with 0.1 mM Cd: I, PC2; II, MT1_HL; and III, unknown.

Results

The induction of thiol-containing compounds in *H. lugdunensis* strain H8-2-1 under Cd-stress was investigated by reversed-phase HPLC followed by post-column derivatization with the Ellman reagent. As shown in the HPLC elution profile (Fig. 1), an increase of the glutathione content, as well as the induction of three further compounds was observed after Cd exposure of the fungus. As the negative control without DTNB did not show any peak, these compounds are Ellman positive and therefore assumed to contain sulfhydryl groups. Fractions I and II (Fig. 1B) were collected before Ellman derivatization and analyzed by mass spectrometry. In fraction I, the singly charged molecular ion $[M + H]^+$ at *m/z* 540.1 was detected and the ESI-MS/MS fragment ion spectrum confirmed a sequence belonging to PC2 ($[\gamma$-Glu-Cys$]_2$-Gly) with complete *y*- and *b*-series (data not shown). Comparable fragmentation patterns of PC2 were published for *S. pombe* [31] and *S. cerevisiae* [26].

In fraction II (Fig. 1B), a second Ellman-positive compound induced by Cd was found with a molecular mass of 2329.7 Da. The MALDI-PSD fragment ion spectrum of *m/z* 2329.7 displayed a long series of *y*-ions (y_1 to y_{14}) due to a C-terminal histidine (Fig. 2A) The ESI-MS/MS spectrum was more complicated, which however showed some *b*-ions (b_3 to b_7) (Fig. 2B). Edman sequencing of the N-terminus finally completed the sequence **SPCTCSTCNCAGACNSCSCTSCSH** (Fig. 2). In the mass spectrum of fraction III (Fig. 1B), no significant ions could be detected.

In order to maintain the intact Cd protein complex and to exclude acid proteolysis, the non-acidified extract of *H. lugdunensis* strain H8-2-1 was separated by SEC (Fig. 3A). In the MALDI MS of fraction III (Fig. 3A), showing characteristic UV absorption of the Cd-sulfide bond at 254 nm (data not shown), three ions with *m/z* 2321.5 (apo-MT), *m/z* 2431.5, and *m/z* 2541.5 were detected (Fig. 3B). The formation of 4 disulfide bridges produced a mass difference of 8 Da in comparison to the molecular mass of the apo-MT in acidic solution. The other masses correspond to chelating one and two Cd ions via Cys-Xaa-Cys and Cys-Xaa-Xaa-Cys motifs.

HPLC fractionation and subsequent MS analyses showed that the fungus is capable of synthesizing both PC2 and MT in response to 0.1 mM Cd.

Discussion

The integrated activity of glutathione, phytochelatins, and metallothioneins in fungi during heavy metal stress is not fully understood. The aim of the present study was: (a) to measure the thiol peptide induction under Cd stress and (b) to develop a method for simultaneous separation and unequivocal characterization of such compounds.

Cd exposure (0.1 mM) increases the glutathione content in the aquatic hyphomycete *H. lugdunensis* (Fig. 1). The internal glutathione pool of the mycelium increased under 0.1 mM Cd from 8 μmol g dw^{-1} (control) to 18 μmol g dw^{-1} (B. Braha, unpublished). The increased GSH content could be explained by the higher requirement due to its function as a precursor of the PC synthesis. Moreover, the formation of Cd-GSH complexes can be assumed.

We hypothesize that a substantial proportion of reduced glutathione is removed from the glutathione pool under Cd stress to detoxify the toxic metal by intracellular chelation. In *C. glabrata*, intracellular Cd complexes

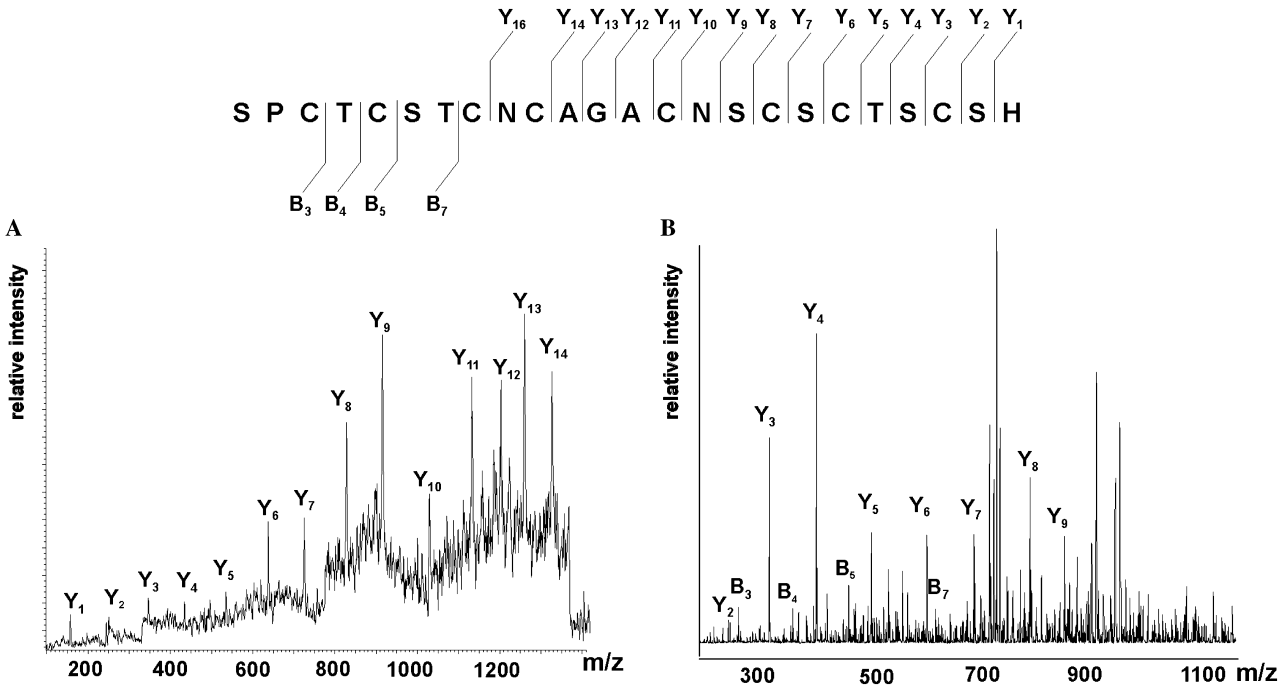

Fig. 2. Sequence analysis of compound II according to Fig. 1B by mass spectrometry: (A) MALDI-PSD MS spectrum; (B) ESI-MS/MS; inset: *y*-ions due to a C-terminal histidine. Sequence was finally completed by Edman degradation.

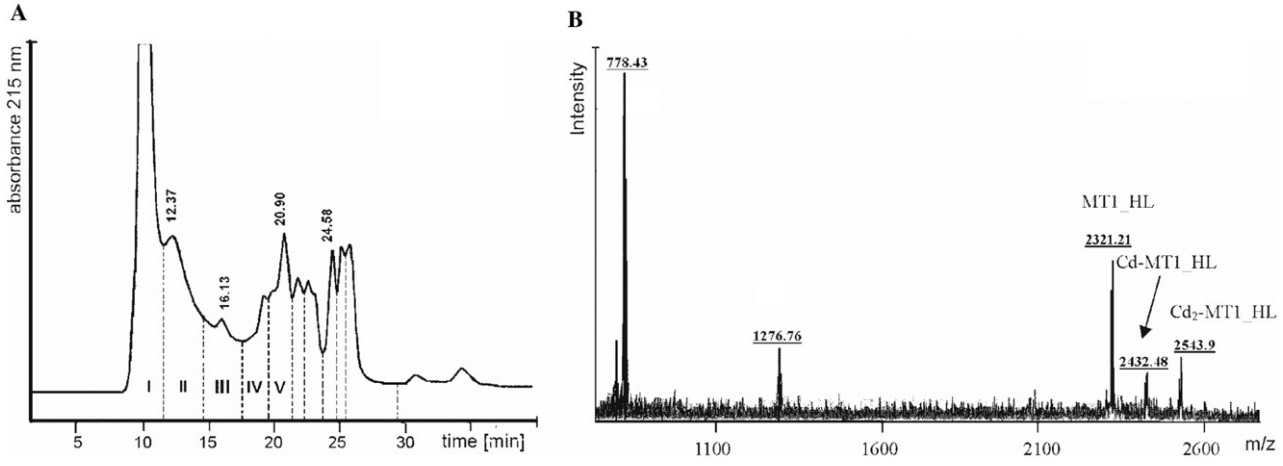

Fig. 3. (A) SEC fractionation of the extract from *H. lugdunensis* strain H8-2-1. The extract was not acidified in order to maintain the intact Cd(II)–protein complex. Chromatographic conditions compared to Materials and methods. (B) MALDI mass spectrum of the peak III in (A), representing MT1_HL from *H. lugdunensis*. All cysteines form disulfide bridges.

were gradually formed with increasing exposure, but Cd–glutathione complexes were the first to appear [32]. In *S. cerevisiae*, Cd detoxification is mainly dependent on binding by glutathione as bis(glutathionato) Cd and the sequestration of these complexes into the vacuole mediated by the ABC-transporter YCF1 [33]. Some evidence for intracellular metal chelation by GSH was given for Cd stressed fungus *Paxillus involutus* by: (a) the remarkable increase of glutathione and its precursor γ-glutamyl-cysteine, accompanied by the complete absence of PCs [34], and (b) the high Cd content in vacuoles [35], possibly mediated by a bis(glutathionato) Cd transporter.

In aquatic hyphomycetes, induction of PC during the logarithmic growth phase has not been described before. Other than PC2, no higher molecular PCs are induced in *H. lugdunensis*. In response to Cd the fungi *S. cerevisiae* and *N. crassa* also synthesize PC2, only [26]. The formation of Cd–PC2 complexes by *H. lugdunensis*, as described for *S. pombe* [36], remains to be investigated.

Surprisingly, a novel MT was induced simultaneously with PC2 induction in *H. lugdunensis* under Cd stress. The

Table 1
Comparison of the sequences of the Cys-rich MT from *H. lugdunensis* strain H8-2-1 (MT1_HL) and MT of family 8 (http://www.expasy.ch/cgi-bin/lists?metallo.txt), *C. gloeosporioides* (MT1_COLGL and MT2_COLGL) [34], *A. bisporus* (MT_AGABI) [35], and *N. crassa* (MT_NEUCR) [30]

Entry name	Accession number	Sequence information
MT1_HL		-SPCTCS---TC--NCAGACNSCSCTSCSH- 2330 Da
MT2_COLGL	Q00369	MAPCSCK---SCGTSCAGSCTSCSCGSCSH- 2549 Da
MT1_COLGL	Q99334	MSGCGCASTGTC--HCGK---DCTCAGCPHK 2519 Da
MT_AGABI	P04358	-GDCGCSGASSC--TCAS--GQCTCSGCGK- 2233 Da
MT_NEUCR	P02807	-GDCGCSGASSC--NCGS--G-CSCSNCGSK 2234 Da

UniProtKB/Swiss-Prot entry primary accession number (accession number).

content of 33% cysteine as well as Cys-Xaa-Xaa-Cys and Cys-Xaa-Cys sequence motifs are characteristic of metallothioneins. To the best of our knowledge, MALDI-PSD was used for the first time to sequence such compounds. Apart from the high cysteine content the MT sequence contains a noticeable amount (40%) of hydroxy amino acids serine and threonine. We hypothesize that these amino acids may supplement the action of cysteine and also contribute to heavy metal chelation. Detoxification of Cd by MTs can be expected, because binding of at least two Cd ions by the novel MT was demonstrated in the MALDI mass spectrum (Fig. 3B). An in vivo bonding of three Cd cannot be excluded, because the loss of more loosely complexed Cd ions, e.g., by carboxylate bonding is possible during the acquisition of the MALDI mass spectrum. Spectroscopic studies suggest that the *N. crassa* MT coordinates a third metal ion in a different manner by metal sulfur coordination [37].

The *H. lugdunensis* MT appears to be the first MT described which is induced by Cd together with a PC accompanied by an increase in GSH level. It remains to be seen whether the three peptides all function in metal buffering in fungal cells. The novel MT isolated from *H. lugdunensis* can be categorized as a member of the MT superfamily and was designated as MT1_HL according to the Swiss Protein Knowledgebase (http://www.expasy.ch/cgi-bin/lists?metallo.txt) [4]. MT1_HL is assigned to family 8 (fungi-I MTs) [Vašák, personal communication] and shows with 60% identity the nearest sequence to MT2_COLGL (http://www.expasy.ch/cgi-bin/lists?metallo.txt) [4] from the plant pathogenic fungus *C. gloeosporioides* [18] (Table 1). In this fungus, Northern blot analysis showed the presence of Cd and Cu induced larger amounts of transcripts, coding for MT2_COLGL, than for transcripts coding for MT1_COLGL. But gene encoded proteins were not isolated [18].

Some identity in amino acid sequences exists to other small MTs from *N. crassa* [38] and the basidiomycete *Agaricus bisporus* [39] (Table 1).

The method developed allows the simultaneous detection of heavy metal-binding thiol peptides in Cd exposed *H. lugdunensis*. The integrated use of modern methods for separation and structure analysis of peptides and proteins are prerequisites for a more detailed characterization of response to heavy metals. Forthcoming studies will explore the potential role of the thiol peptides glutathione, PC2 and MT_HL1, as natural biotools for detoxifying heavy metals and environmental sensing.

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