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Heavy Metal Induced Total Proteins Profile of Ciliates Isolated from Industrial Wastewater

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Abstract.- PAGE analysis of total proteins of the protozoans showed that a number of proteins were induced when the ciliates were exposed to Pb^{2+} (4 µg/ml), Cr^{6+} (4 µg/ml) and Cd^{2+} (2 µg/ml). Nine protein bands (4 µg/ml) (70kDa, 60kDa, 50kDa, 45kDa, 40kDa, 38kDa, 23kDa, 20kDa and 18kDa) appeared after Pb^{2+} treatment, seven bands (4 µg/ml) (60kDa, 50kDa, 45kDa, 40kDa, 20kDa, 18kDa and 14kDa) appeared after Cr^{6+} treatment, and six bands (2 µg/ml) (50kDa, 45kDa, 40kDa, 30kDa, 18kDa and 9kDa) appeared after Cd^{2+} treatment. Certain proteins disappeared as a result of metal stress. For example 55kDa and 44kDa proteins, which were present in the control, Pb^{2+}-treated and Cr^{6+}-treated samples but were absent in the Cd^{2+} treated sample. It was observed that few proteins with low molecular weights were induced in *Paramecium caudatum* (23kDa, 18kDa), *Vorticella microstoma* (22kDa, 20kDa, 9kDa), *Oxytricha fallax* (12kDa, 9kDa), and *Euplotes mutabilis* (20kDa, 18kDa, 15kDa) against metal stress which indicates that nearly all protozoans produce MT like proteins which may be involved in heavy metal handling and detoxification processes.

Key words: Ciliates, heavy metal; metal detoxification; metallothionein.

INTRODUCTION

Protozoans just like other microorganisms cope with high concentration of toxic compounds including heavy metals in the medium by a variety of mechanisms - synthesis of metallothionein (MT) proteins being one of the several responses. It has been found that ciliated protozoans exposed to heavy metals are able to synthesize MTs (Piccinni *et al*., 1994, 1999; Santovito *et al*., 2000; Boldrin *et al*., 2002; Martin-Gonzalez *et al*., 2006), suggesting that also in these organisms, MTs play a role in heavy metal homeostasis and detoxification.

Metallothioneins (MTs) represent a family of small, cytosolic, cysteine-rich polypeptides whose synthesis is enhanced by heavy metal tissue accumulation (Hamer, 1986; Kägi and Kojima, 1987; Kägi, 1993). Primary structure of MTs shows a lack of aromatic residues and a high content of cysteines (about 305 of the total amino acid content), which are allocated in motifs such as Cys-X-Cys, Cys-XY-Cys (where X and Y are amino acids different from Cys) and Cys-Cys (Kägi and Kojima, 1987; Kägi, 1993). This typical distribution of residues confers, through the formation of two distinct metal tetrathiolate clusters (Kägi and Kojima, 1987), a high binding capacity for essential heavy metal ions, such as zinc and copper and for cadmium, mercury and silver which, on the contrary, are very toxic to the cell. These MTs play important role in the regulation of essential metals and detoxification of the unusual entry of essential and nonessential metals (Hamer, 1986; Roesijadi, 1992; Culotta *et al*., 1994; Vasak, 2005; Rigby *et al*., 2006). Large parts of MT-related studies have focused on the potential of using MTs as specific biomarkers of heavy metal exposure, because induction of MT within some target tissues has been known to be clearly elevated after animals were exposed to metals (Roesijadi, 1992; Pederson *et al*., 1997; Kameo *et al*., 2005; Damiens *et al*., 2006).

A cadmium binding protein (23.6kDa) with biochemical features of MTs has been isolated from the *Tetrahymena thermophila* (Dondero *et al*., 2004). Thioredoxin gene expression in *Chlamydomonas reinhardtii* has been implicated in the defense mechanism against heavy metals (Lemaire *et al*., 1999). Zimeri *et al*. (2005) reported that MT1 are preserved in plant genomes due to their distinct metal binding properties and confers

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tolerance to cadmium and assists in zinc homeostasis.

MTs have been isolated from many sources including protozoa such as *Oxytricha* and *Tetrahymena* (Piccini *et al*., 1994; Irato *et al*., 1995). However, in this paper we describe the evidence of the presence of an MT-like protein in ciliates of environmental importance and its possible role in resistance to heavy metals.

MATERIALS AND METHODS

Sampling, culturing and treatments

Water samples were collected, in sterilized screw capped glass bottles, from tanneries of industrial area of Kasur, a small town near Lahore city (Pakistan). The sampling site comprised five different wastewater ponds receiving effluents from approximately 500 tanneries located near the Leather Service Station of Punjab Small Industries (Kasur). The isolated ciliates described in this report have been now maintained in the culture in the lab for the last 4 years.

Axenic culture of protozoa *viz*., *Vorticella microstoma, Oxytricha fallax, Stylonychia mytilus, Euplotes mutabilis* and *Paramecium caudatum* was prepared according to Shakoori *et al*. (2004). One hundred milliliters of Bold-basal salt medium [$NaNO_3$ (0.25g/L), $CaCl_2.H_2O$ (0.025g/L), $MgSO_4.7H_2O$ (0.075g/L), K_2HPO_4 (0.075g/L), KH_2PO_4 (0.175g/L), NaCl (0.0025g/L), EDTA (0.05 g/L), KOH (0.031g/L), $FeSO_4$ $.7H_2O$ (0.04g/L), H_2SO_4 (0.001,L/L), H_3BO_3 (0.01142g/L), $ZnSO_4.7H_2O$ (0.00881g/L), $MnCl_2.4H_2O$ (0.00144 g/L), MoO_3 (0.00071g/L), $CuSO_4.5H_2O$ (0.00157 g/L) and $Co(NO_3).6H_2O$ (0.00049g/L)], diluted 1:1000 with distilled water, in 250 ml conical flask, was inoculated under aseptic conditions with 10µl of inoculum containing 40-50 ciliates. Glucose, as carbon source, was added as 1g/L in Bold-basal salt medium (Shakoori *et al*., 2004; Rehman *et al*., 2005; Rehman *et al*., 2006). The pH of the medium was adjusted at 7.5. The cultures were maintained in the laboratory at room temperature (25-27°C).

All treatments with heavy metals were performed in Bold-basal salt medium inoculated with 40 protozoan cells in four 250 ml conical flasks, one control and three metal treated (Pb, 4 µg/ml; Cr, 4 µg/ml; Cd, 2 µg/ml), and incubated at 25-27°C for three days.

Isolation of total proteins from protozoa

The cells were harvested at log phase of growth (72-96 hrs) by centrifugation at 9000 rpm (1008 x g) at 4°C for 15 minutes. The pellet was suspended in 1 ml distilled water in an eppendorf tube, to which 200 µl of 10% SDS buffer (10% SDS, 0.001% mercaptoethanol) was added. The tubes were vortexed and centrifuged at 10,000 rpm (1120 x g) for 5 minutes. The supernatant was discarded, and the pellet was resuspended in distilled water, heat shocked in boiling water bath for 5 minutes and then immediately transferred to ice box for 2 minutes. Tubes were vortexed and heat shocked again for 3 minutes. Now tubes were centrifuged for 30sec. at 10,000 rpm (1120 x g) and finally supernatant was removed and stored at -80°C. Total protein concentration was determined according to the Lowry method (Lowry *et al*., 1951) with the help of a standard curve using 50, 100, 150, 200, 250, 300 and 350 µg/ml of bovine serum albumin per 0.4 ml of saline solution (0.5 M). Extra care was taken to process the sample as soon as possible, without letting sample stay outside ice.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Protein profile of the protozoa was analyzed by 12% PAGE as described by Laemmli (1970). Finally 20 µg of protein were directly subjected to 12%sodium dodecyle sulphate (Acrylamide 6 ml, Tris HCl 3.8 ml, pH 8.8, 10% Ammonium persulphate, 50µl, 12% SDS, 50µl, TEMED 10µl) polyacrylamide gel electrophoresis. The gels initially run at 70volts until the sample had passed through the stacking gel and reached the resolving gel, and then the current was changed to 100volts for 4 hours at room temperature. Coomassie blue was used for staining the gel. After overnight destaining, the gels were photographed.

RESULTS

Figures 1 and 2 show electrophoretic pattern of proteins of different ciliates isolated from industrial wastewater after induction with lead, chromium and cadmium.

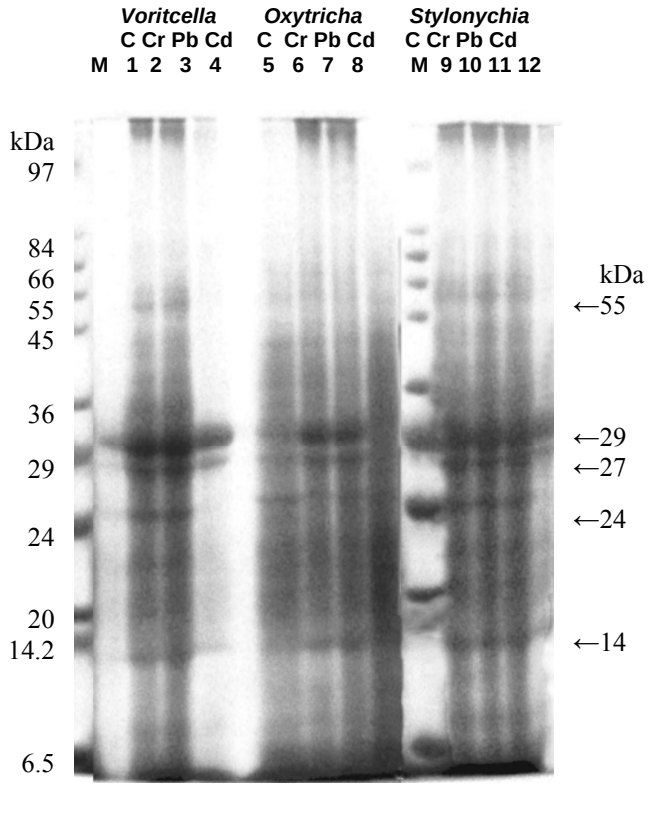

Fig. 1. SDS-PAGE pattern of total proteins in *Vorticella microstoma* (lanes 1-4), *Oxytricha fallax* (lanes 5-8) and *Stylonychia mytilus* (Lanes 9-12). M, protein markers; Lanes 1, 5 and 9, control (non-treated); Lanes 2, 6 and 10, Cr treated; Lanes 3, 7 and 11, Pb treated; Lanes 4, 8 and 12, Cd treated. The gel is 12%, stained with Coomassie blue.

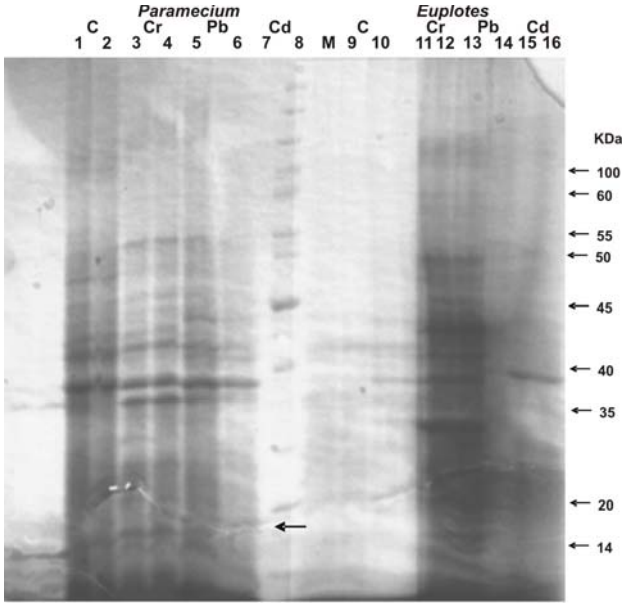

Fig. 2. SDS-PAGE pattern of total proteins in *Paramecium caudatum* (lanes 1-8) and *Euplotes mutabilis* (lanes 9-16). M, protein marker; Lanes 1, 2, 9 and 10, control (non-treated); Lanes 3, 4, 11 and 12, Cr treated; Lanes 5, 6, 13, and 14, Pb treated; Lanes 7, 8, 15 and 16, Cd treated. The gel is 12%, stained with Coomassie blue.

Vorticella microstoma

PAGE analysis showed that a number of new protein bands with molecular weights of 55kDa, 45kDa, 40kDa, 36kDa, 22kDa and 20kDa were induced in *Vorticella microstoma* when Cr and Pb were added to the culture medium but these bands were absent from Cd treated sample. Some proteins with molecular weights of 29kDa, 27kDa, 24kDa and 14kDa were present in all samples but their intensity was greater in metal treated samples as compared to control sample. Two proteins of 84kDa and 9kDa were present in all metal treated samples but was less prominent in Cd treated sample and was absent from control sample (Fig. 1).

Oxytricha fallax

In *Oxytricha* ten proteins with molecular weights of 66kDa, 55kDa, 45kDa, 40kDa, 36kDa, 29kDa, 27kDa, 24kDa, 20kDa and 14kDa were present in all samples but comparatively highly expressed in metal treated samples as compared to control. Two proteins of 12kDa and 9kDa molecular weights in *Oxytricha* were induced in metal treated samples but these bands were absent in control sample. Two proteins of 23kDa and 22kDa were also visible in control and treated samples (Fig. 1).

Stylonychia mytilus

PAGE of *Stylonychia* showed that there were 4 prominent protein bands in the control (untreated) and metals treated samples with molecular weights of 29kDa, 27kDa, 14kDa and 9kDa but 14kDa and 9kDa protein bands were less prominent in Cd treated samples as compared with control. Seven protein bands with molecular weights of 55kDa, 45kDa, 40kDa, 36kDa, 24kDa, 22kDa and 20kDa were also present in control (untreated), Cr and Pb

treated samples but absent from Cd treated sample (Fig. 1).

Paramecium caudatum

Only two proteins were visible in the untreated (control) sample of *Paramecium* with molecular weights of 38kDa and 15kDa which were also present in metal treated samples with higher expression. A new protein of 70kDa was induced in metal treated samples but was absent from control sample. Another eight new proteins of 60kDa, 50kDa, 47kDa, 45kDa, 40kDa, 35kDa, 30kDa and 23kDa were induced in metal treated samples in *Paramecium* but were absent in control sample. A new low molecular weight protein of 18kDa was also induced in metal treated samples but was absent in control sample (Fig. 2).

Euplotes mutabilis

Addition of metals into the growth medium of *Euplotes* cells resulted in expression of some proteins. In *Euplotes* seven new proteins of 60kDa, 55kDa, 50kDa, 45kDa, 35kDa, 20kDa and 18kDa were induced when Pb was added to the culture medium. A new protein with molecular weight of 38kDa was induced in metal treated samples but was absent from the control. Three other proteins with molecular weights of 20kDa, 18kDa and 15kDa were induced in *Euplotes* with the addition of Pb and Cd treated samples but were absent in Cr treated and control samples. A protein with molecular weight of 44kDa was present in control, Cr and Pb treated samples but this protein was absent in Cd treated sample (Fig. 2).

DISCUSSION

In order to check the effect of metal on the production of proteins a comparison was carried out among control and chromium, cadmium and lead treated ciliates through PAGE. It was found that new proteins were induced under metal stress (*e.g.,* 70 kDa protein in *Paramecium,* 60kDa protein in *Euplotes* and 45kDa protein in *Vorticella* against Pb^{2+}) appeared in the treated ciliates indicating that proteins were produced under stress conditions. Besides the production of specific proteins, there was also reduction of bands in the treated (*e.g.,* 55kDa protein was present in control but no such band was observed in Cd treated sample in *Stylonychia*) as compared with control indicating the toxic effect of metal on protein machinery of resistant ciliates.

A low molecular weight (15kDa) protein was induced in *Euplotes* when Cd and Pb separately were added to the culture medium. Similar result (12kDa protein) was obtained from another ciliate, *Oxytricha*, when metals were added to the culture medium. Presence of such low molecular weight metallothionein like proteins in ciliates indicates that these organisms are adapted to produce such proteins during metal stress conditions.

In the present study almost some similar proteins were observed with molecular weight of 23kDa protein in *Paramecium caudatum* against Cr and Pb, 20 kDa protein in *Vorticella microstoma* against Cr and Pb and 12kDa protein in *Oxytricha fallax* against Cr, Pb and Cd which indicates that nearly all protozoans produce MT like proteins which may be involved in heavy metal handling and detoxification processes.

Ting *et al*. (2005) reported that *MTT1* gene in *Tetrahymena thermophila* was more sensitive to cadmium induction compared to copper at a concentration of ≤35.2 µmol/l. Furthermore the *MTT1* expression level increased when cadmium concentration was increased. At a concentration lower than 22 µmol/l, cadmium cooperated with copper to increase the expression level of *MTT1*, hence enhancing the detoxification ability of *Tetrahymena thermophila*.

Roesijadi (2000) described active role of MTs in the protective response to metal toxicity. The efficacy of such a mechanism for metal detoxification has been demonstrated with enzymes, actin and zinc finger proteins. With zinc finger proteins, zinc MT can restore both altered secondary structure and inhibited DNA-binding function to functional states through zinc for cadmium exchange. The presence of heavy metals activates the transcription of MT genes via the binding of metal-binding regulatory factors (MRFs) to the metal-responsive elements (MREs) (Roesijadi, 1992; Wu *et al*., 2006).

Shakoori *et al.* (2000) reported that besides production of specific proteins there was reduction

of bands in lead treated yeasts as compared to untreated controls. This represents the toxic effect of metals on protein production.

Comparison of PAGE pattern of proteins of control and treated protozoans showed that some proteins that exist in the control are absent in the treated protozoans (*e.g*., 55kDa protein was present in control but no such band was observed in Cd treated sample in *Stylonychia*) indicating switching off of protein synthetic machinery (Shakoori *et al*., 2000). Besides reduction of specific proteins, there was also induction of some new proteins in the treated group (*e.g.,* 50kDa protein in *Paramecium*, 38kDa protein in *Euplotes* and 36kDa protein in *Vorticella* were only present in metal treated samples but were absent in control sample). This indicates expression of some specific genes under metal stress.

Martin-Gonzalez *et al*. (2005) reported that the appearance of electron dense accumulations in the cytoplasm, which might be related to MT-mediated detoxification. Boldein *et al*. (2002) reported the presence of two different MTs in *Tetrahymena pigmentosa*, induced specifically by copper and cadmium. The presence of a cadmium-inducible MT gene in cells of *Tetrahymena thermophila* has been demonstrated (Shang *et al*., 2002).

At present, the purified and characterized MTs in ciliated protozoa are only those from *Tetrahymena pyriformis*, *T. pigmentosa* and *T. thermophila*. In addition, several genes encoding Cd-MTs have been isolated recently in other species of ciliates, such as *Colpoda inflate, Uronema nigricans* and *Oxytricha nova* (Diaz *et al*., 2001). Cu-MTs have also been identified in *T. thermophila* and *T. pigmentosa*; they are shorter and quite different from the Cd-MTs isolated from ciliates (Santovito *et al*., 2001). PAGE analysis of proteins in different ciliates showed that some proteins that exist in all ciliates (*e.g.,* 60kDa, 50kDa, 45kDa, 40kDa, 35kDa and 20kDa proteins were common to both *Paramecium* and *Euplotes*) and some proteins present in one organism (*e.g.,* 70kDa protein was visible in *Paramecium* but not in *Euplotes*). Likewise another 25kDa protein was present in *Euplotes* but was not seen in *Paramecium* (Fig. 2). This indicates that protein response of the ciliates was metal as well as organism specific.

Boldrin *et al*. (2006) reported a new MT gene from *Tetrahymena thermophila* that has a strong promoter induced by an essential metal, Cu. This promoter can be turned on and off rapidly, making it a good system for induction of ectopic gene expression in *Tetrahymena thermophila* and enhancing its applications in cell and molecular biology, as well as biotechnology. Because *MTT2* gene expression requires Cu rather than Cd, its promoter may offer an attractive alternative to the previously described *MTT1* promoter for driving foreign gene expression in *Tetrahymena thermophila* and clearly suggest that the upstream region of *MTT2* can direct high-level expression of heterologous genes.

Ciliates are good candidates for use as whole-cell biosensors to detect the presence and to determine the bioavailable concentration of heavy metal ions in natural samples. In bioaccumulation, metallic intracellular detoxification includes the participation of MTs that bind the metal ions. The synthesis of MTs by the ciliates, when exposed to metal ions, during the present study indicates that these ciliates have excellent metal uptake ability and can be used in the development of a strategy for better exploitation of protozoans for the treatment of toxic wastewater released by the industries. Therefore, use of these eukaryotic microorganisms in bioremediation studies as potential whole cells or molecular (ciliate metallothioneins) would seem to be a reasonable and useful approach for assessing metallic pollution.

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