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Journal of Chromatography B, 820 (2005) 205–210
JOURNAL OF CHROMATOGRAPHY B
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One-step purification of metallothionein extracted from two different sources

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Received 1 October 2004; accepted 14 March 2005
Available online 25 April 2005

Abstract

We describe a one-step purification of hepatic metallothionein from the Amazon fish *Colossoma macropomum* injected with cadmium and from the copper-loaded metallothionein from the yeast *Saccharomyces cerevisiae*, performed by affinity chromatography through metal-chelating columns. Yeast metallothionein was purified from Cu^{2+}-loaded resin and eluted by a continuous EDTA gradient whereas hepatic metallothionein extracted from fishes was purified by Ni^{2+}-loaded resin and eluted by a continuous imidazol gradient. Purified metallothioneins were evaluated by SDS–PAGE and characterized by UV spectra of the apo- and Cd^{2+}-loaded protein. This method allowed high purity and yield as well as rapid one-step extraction of both metal-loaded and apoprotein.

Keywords: Metallothionein; Environmental sciences; Ni and Cd binding; Metal-chellating affinity chromatography

1. Introduction

Metallothioneins (MT) are a superfamily of low molecular weight cysteine-rich proteins firstly described in equine kidney by Margoshes and Vallee [1]. They have a widespread expression throughout all eukaryotes as well as in some prokaryotic species. All vertebrates contain two or more MT isoforms [2]. Their function is associated to the high d^{10} metal ions binding capacity though a primary role has not been identified. The two most widely expressed isoforms in vertebrates, MT-1 and MT-2, are rapidly induced in the liver by a wide range of metals, drugs and inflammatory mediators. The thiolate cluster structure of these proteins is involved in the metabolic regulation via Zn^{2+} donation, protection against oxidative damage, and sequestration and/or redox control [3]. An important property assigned for MTs refers to their role in transition metal detoxification (especially Cd^{2+}). It may be noted that in higher organisms, MTs are the sole proteins in which cadmium accumulates naturally. On that regarding MTs have been proposed as biomarkers for environmental control and occupational diseases [4].

It is noteworthy by literature that methods for metallothionein purification described thus far generally include several combined chromatographic steps, such as: gel filtration, ion exchange and HPLC [5–7]. Those methods take time for purification besides the high loss of protein mass along the entire procedure when compared to most procedures by affinity chromatography. Indeed, the extraction and purification of native proteins usually involve several steps unless the protein possesses some peculiar structural characteristic or physical–chemical property that allows a special procedure for purification. In that regard, metallothioneins present a cysteine-rich primary structure. As well known, cysteine is prone to chelate transition metals. Based on that property, in the present work, we describe a simple method for pu-

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doi:10.1016/j.jchromb.2005.03.017

rification of metallothioneins from two different sources by affinity chromatography through metal-chelating columns.

Our goal to conduct the experiments described in the present report was to develop a simple method to isolate metallothionein from tissues of fishes since our major interest will be the study upon the role of metallothionein from some Amazon fish species as biomarkers of metal-polluted waters, as already described for other species from different environments [12–15].

2. Conditions

2.1. Reagents

Bovine Serum Albumin, Bradford Reagent and cadmium, nickel or copper salts were purchased from Sigma (Saint Louis, LO, USA). The 1-mL HiTrap™ Chelating HP column and protein molecular mass markers, Rainbow™ and Broad Range PMWS™, were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents utilized were of analytical grade and the water was purified with the Milli-Q system from Millipore.

2.2. Animals and cadmium treatment

Specimens of tambaqui (*Colossoma macropomum*) were collected from floodplains in Marchantaria island (3°15′S 59°58′W). The animals were maintained in 2000-L tanks at 25 °C with aerated fresh water and fed ad libitum for one week. After this period, the animals ($n = 6$) were weighted (319.38 ± 38.98 g), measured (21.13 ± 0.62) and injected intra-peritoneally with $CdNO_3{\cdot}4H_2O$ dissolved in 50 mM sodium phosphate buffer, pH 7.4, to a final concentration of 3 μg $CdNO_3{\cdot}4H_2O$/g fish. Six animals were utilized for the control group injected with similar volumes of buffer solution without cadmium. The animals were maintained individually into aerated 20-L fishbowls for 48 h. Afterwards, the liver was extracted and homogenized (35% w/v) in 50 mM Tris–HCl, pH 7.4; containing 0.1 mM PMSF, 0.5 mM DTT and 150 mM NaCl in a Teflon homogenizer. The homogenate was centrifuged at 15,000 × *g* for 90 min at 4 °C. The supernatant was immediately applied to a HiTrap™ Chelating HP column previously loaded with $NiSO_4{\cdot}6H_2O$, as described below.

2.3. Yeast growth and metallothionein extraction

We utilized a strain of *Saccharomyces cerevisiae* belonging to the ExClone™ collection (yeast ORF expression clones; ResGen, Invitrogen Corp., UK), transformed with a plasmid carrying an open reading frame for the yeast metallothionein CUP1A. Cells were cultured in synthetic minimum medium (2% glucose and 2% peptone, supplemented with amino acids, except leucine and uracyl base) at 30 °C with reciprocal shaking to an optical density of the culture at 600 nm of 0.8. At that point, 0.5 mM $CuSO_4{\cdot}5H_2O$ was added to the culture medium and cells were incubated for further 2 h in the same conditions described above. Afterwards, cells were harvested and pelleted by centrifugation. The pellet was washed twice with extraction buffer (50 mM Tris–HCl, pH 7.4; containing 0.1 mM PMSF, 0.5 mM DTT and 150 mM NaCl). Yeast cells were disrupted according to the protocol described by Verma et al. [8]. After centrifuged at 15,000 × *g* for 90 min, the cell extract was applied to a HiTrap™ Chelating HP column previously loaded with $CuSO_4{\cdot}5H_2O$, as described below.

2.4. Purification of metallothionein

Affinity chromatography was performed using a 1-mL HiTrap™ Chelating HP column attached to a P1 peristaltic pump (Amersham Pharmacia Biotech). Supernatants from yeast cells and from the liver of fishes obtained as described above were applied to the column previously loaded with $CuSO_4{\cdot}5H_2O$ or $NiSO_4{\cdot}6H_2O$, respectively. After metal loading, the column was equilibrated with 10 volumes 50 mM Tris–HCl, pH 7.4 added of 0.15 M NaCl, here called equilibrating buffer. After applying the supernatants, 10 volumes of equilibrating buffer were passed through the column. The elution was performed next, either by 5 mL of continuous gradient of EDTA (0–0.1 mM) buffered in 50 mM Tris/HCl, pH 7.4 (MT extracted from yeast homogenates) or by 10 mL of continuous gradient of imidazol (0–500 mM) for the extraction of MT from liver homogenates of fish. Imidazol was dissolved in 50 mM Tris–HCl, pH 7.4 added of 0.15 M NaCl. In both extractions (yeast or liver homogenates), fractions (1 mL) were collected at a flow rate of 1 mL/min through the entire procedure. The purification was performed at room temperature. Once finished the elution procedure, the column was washed with 10 volumes of 1 mM EDTA (this step is called washing-step), followed by 20 volumes of Milli-Q fresh water to recover the column, according to the protocol enclosed to the manufacturer's technical data sheet. Aliquots (20 μL) of each fraction obtained from the elution step were applied to 15% SDS–PAGE. Metallothionein-positive fractions were deduced on the gel by the molecular mass, spectral characterization and metal determination, as described below and shown in the figures. Metallothionein-positive and purest fractions were combined to prepare the apoprotein (apoMT).

2.5. Preparation and characterization of ApoMT

ApoMT was obtained by the incubation of metallothionein-positive fractions, obtained as described above and deduced from SDS–PAGE, with 50 mM DTT and 10 mM HCl solution, according to Dallinger et al. [9]. The preparation was applied to a G-25 (Sigma) column (20 cm × 1 cm) pre-equilibrated with 10 mM HCl and 10 mM NaCl. One-millilier fractions were collected starting at sample application. The entire procedure to obtain apoMT was performed under helium atmosphere at 4 °C. Fractions obtained were applied

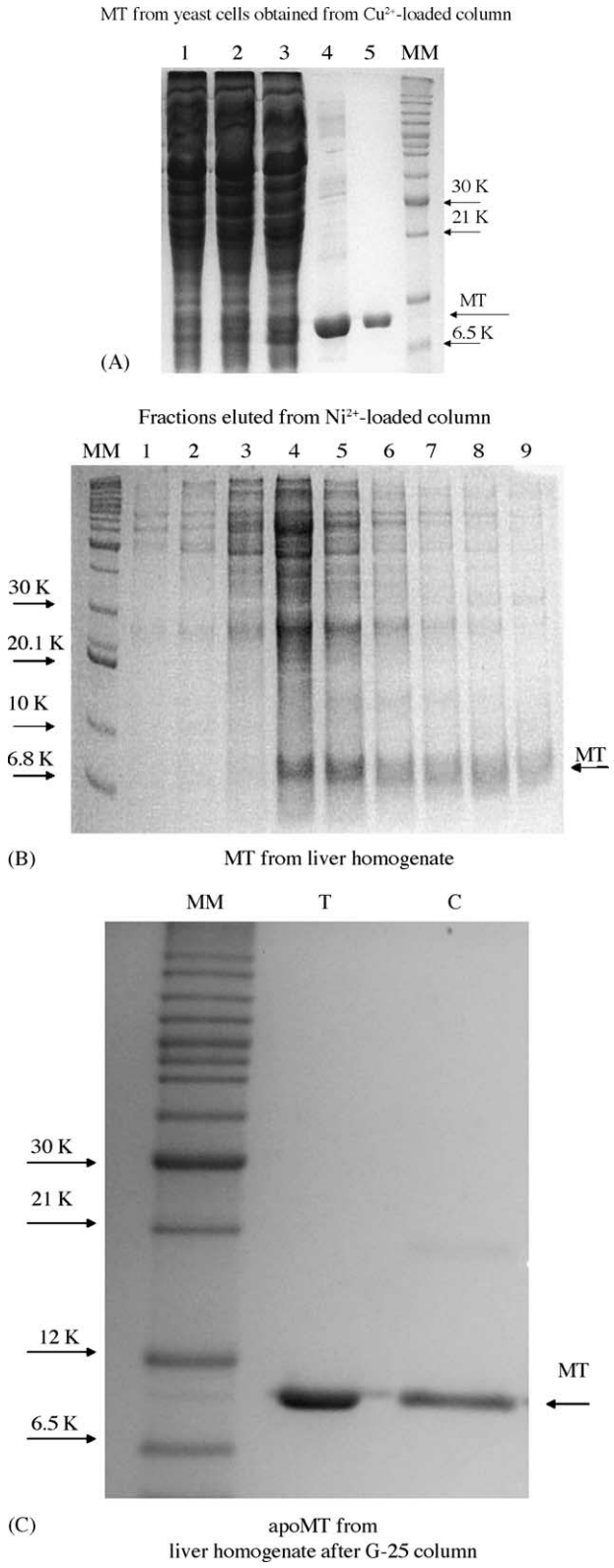

Fig. 1.

to 15% SDS–PAGE under reducing conditions. MT-spectra were obtained from apoMT sole or by adding $CdNO_3{\cdot}4H_2O$ at increasing concentrations (from 8 μM up to 80 μM) to the apoMT aliquots obtained from fish livers [10].

2.6. Ni^{2+} and Cd^{2+} quantification by graphite furnace atomic absorption

Nickel and cadmium quantification was performed by atomic absorption spectrometry in a Perkin-Elmer Analyst 800 equipment according to the manufacturer's technical protocol under Ar atmosphere. Samples were digested by 10% HNO_3 for 12 h at 70 °C. Twenty micro liters aliquots were taken from each sample and analyzed at 232 nm (Ni^{2+}) or 228.8 nm (Cd^{2+}). Standard curves of $NiSO_4{\cdot}6H_2O$ and $CdNO_3{\cdot}4H_2O$ were run in parallel. Results were expressed in μg/mL of the initial solution.

2.7. Polyacrylamide gel electrophoresis (PAGE)

Protein preparations were electrophoresed by SDS/PAGE under reducing conditions by adding 10 mM DTT (final concentration) to the buffer utilized for the sample preparation for gel application [11].

2.8. Determination of protein concentration

Protein concentration was determined by the Bradford reagent method against a bovine serum albumin curve.

3. Results

We performed isolation and purification of metallothioneins from two differenet sources, yeast and fish, to better characterize the method of purification by metal affinity chromatography. Yeast *S. cerevisiae* MT was chosen because in contrast to MTs from vertebrate sources, in which zinc, cadmium and copper can be simultaneously bound, yeast and fungal MTs contain exclusively copper [3].

As depicted in Fig. 1, metallothionein could be purified from both yeast cell extracts (Fig. 1A) or hepatic extracts of fish (Fig. 1B) by utilizing Cu^{2+}- or Ni^{2+}-loaded column, respectively. Fig. 1A shows a 15% SDS–PAGE loaded with

Fig. 1. SDS–PAGE of metallothioneins extraction. (A) CUP1A metallothionein extracted from *S. cerevisiae* cells. Lanes 1–4 refer to samples obtained during the EDTA-elution step performed by applying 5 mL of continuous EDTA gradient (0–0.1 mM EDTA; 1 mL/min). Lane 5 refers to the yeast apoMT obtained as described in Section 2 from the fraction represented by lane 4. (B) Metallothionein extracted from the liver of *C. macropomum*. Lanes 1–9 refer to samples obtained from the elution step started by applying 10 mL of a continuous imidazol gradient (0–500 mM; 1 mL/min). (C) Apometallothionein. The gel is representative of the apoprotein obtained from liver homogenates of control (C) and Cd^{2+}-treated (T) animals. Both extractions were performed at identical conditions. Gels shown are 15% SDS–PAGE. MM: molecular mass marker.

samples from fractions eluted from the Cu^{2+}-loaded column during EDTA (0–0.1 mM) continuous gradient (lanes 1–4). Lane 5 represents an aliquot of the yeast apoMT eluted from the G-25 column. The apoprotein was prepared from samples, as per lane 4, as described in Section 2. As seen, MT was significantly separated from the other proteins through EDTA continuous gradient (0–0.1 mM buffered EDTA at a flow rate of 1 mL/min). The apoMT, lane 5, was evaluated by spectroscopy to confirm MT identity (result not shown).

Fig. 1B is representative of a 15% SDS–PAGE of fractions eluted from the Ni^{2+}-loaded column during an imidazol continuous gradient (fractions 1–9). At the conditions tested (0–500 mM imidazol; flow rate 1 mL/min), metallothionein from hepatic extracts started to be eluted at fraction 4 and fractions such as 6–9, considered the purest as visualized on the gel, were combined and utilized to obtain the apoprotein, as described in Section 2 and exemplified in Fig. 1C.

Gel (15% SDS–PAGE) shown in Fig. 1C refers to samples of the apometallothionein extracted from liver homogenates of control fishes (C) and fishes previously injected with cadmium (T). Samples shown were prepared from fractions obtained as described in Fig. 1B followed by the apoprotein preparation. By comparing gels shown in Fig. 1B and C, one may observe that metallothionein was completely purified from fractions obtained through Ni^{2+}-loaded column after treatment to obtain the apoMT (Fig. 1C). The simplicity of the method, one-step purification avoiding loss of mass protein in the course of protein isolation, allowed the comparison between metal-exposed and control animals. We have not tried yet other cadmium concentrations in order to test the possibility of a larger dose differentiation.

Metallothionein extracted from the liver of the fish *C. macropomum* was characterized by the UV spectra of the apoprotein and of the Cd^{2+}-protein complex, following the methodology described in the literature [10]. As depicted in Fig. 2A, there is no absorbance at 260 nm of apoMT sample whereas the absorbance at 260 nm was proportionally increased by adding Cd^{2+} to apoMT samples (Fig. 2A and B).

We estimated the apparent molecular mass in SDS–PAGE of metallothionein from the liver of the fish *C. macropomum* within 8000 and 9000 Da (Fig. 1B and C). Although estimate molecular mass of MT herein described does not belong to the range where the majority of this class of proteins is found in liver tissues (MT1 and MT2 isoforms), there are references in the literature that some MT from vertebrates are found within higher MM range [16,17].

A criticism found in the literature [18] upon the utilization of metal-chelating chromatography for the isolation of metalloproteins is the possibility of metal sequestration when those proteins pass through the column, so the protein would not bind on it. To test this possibility we measured Ni^{2+} concentration in each 1-mL fraction eluted from the column along the entire procedure. The results obtained from this set of experiments are shown in Fig. 3. As seen, no significant Ni^{2+}-leaking was observed throughout the procedure

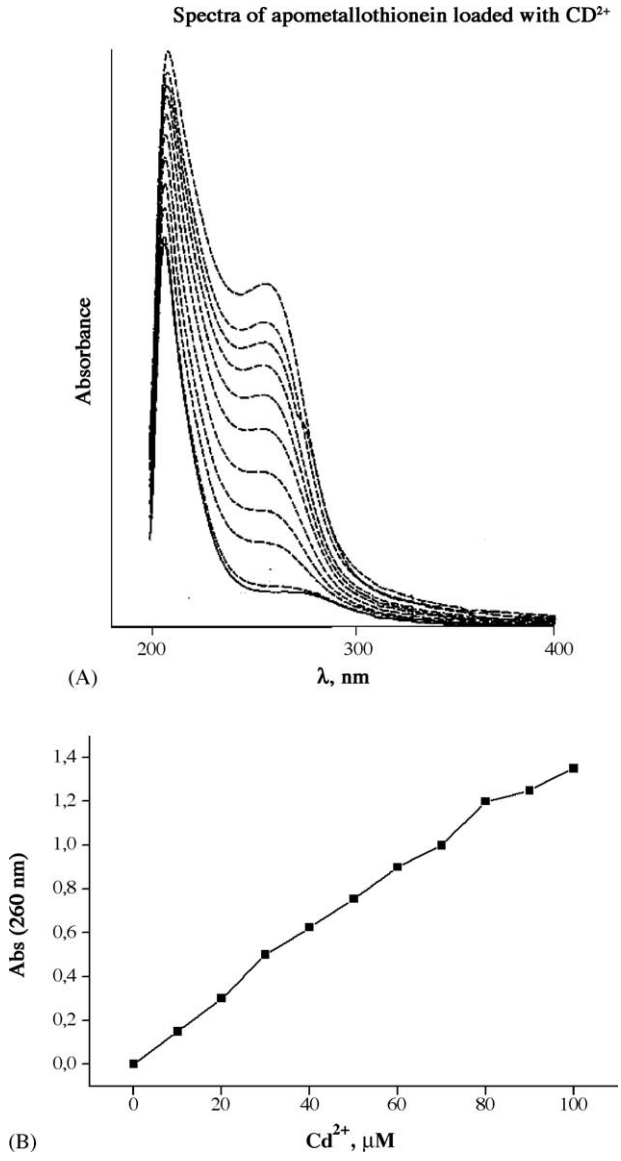

Fig. 2. Spectra obtained from apometallothionein loaded with Cd^{2+}. (A) Spectra shown were obtained with 10 μg/mL apoMT followed by the addition of Cd^{2+} from 8 μM up to 80 μM (final concentration). Solid line represents apoMT. Dashed lines represent Cd^{2+}-loaded samples. (B) Values of absorbance obtained as function of Cd^{2+} concentration were plotted, as shown.

of MT purification (Fig. 3A). Nickle was detected just after loading the column with $NiSO_4{\cdot}6H_2O$ (result not shown) as well as by passing EDTA through the column in order to wash it out (fractions 31–34; Fig. 3A). Nickel concentration was found significantly increased in those fractions where MT was eluted, as shown in the plot of Fig. 3B (fractions 26–28). Cadmium was determined in same fractions in order to further test the identity of the protein eluted from those fractions. Peak of Cd^{2+} concentration was found in the same fractions (27 and 28) referred for Ni^{2+}: 2.0 μg/mL and 3.8 μg/mL, respectively. We also controlled the possibility whether MT bound to the column would not be completely eluted by imidazol. On that, we analyzed by SDS–PAGE

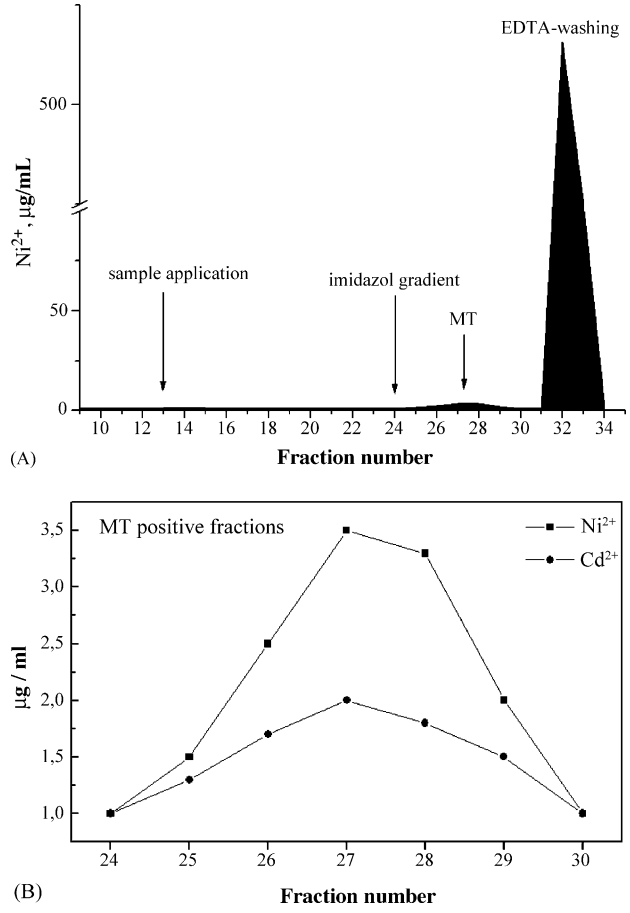

Fig. 3. Evaluation of Ni^{2+}-leaking from the column. Ni^{2+} and Cd^{2+} were quantified by atomic absorption spectrometry as described in Section 2. (A) Aliquots (20 μL) for quantification were taken from 1-mL fractions (flow rate, 1 mL/min) obtained from the entire procedure, starting at column equilibration (fraction number 10) through the end of EDTA-washing step (fraction number 34). (B) Data shown refer to the quantification of Ni^{2+} and Cd^{2+} from MT-positive fractions (24–30) obtained as shown in (A) during the elution by imidazol gradient.

aliquots from the EDTA solution passed through the column during the washing procedure, performed just after imidazol gradient. No protein band from aliquots obtained of EDTA-washing step was detected on the gel (result not shown). Thus, we concluded that the method herein described allows a high pure MT isolation without significant loss of protein mass.

4. Discussion

Our goal for the purification of the MT CUP1A from the yeast *S. cerevisiae* was to test the efficiency of the metal-binding column here utilized for diverse transition metals. We selected the yeast MT because this eukaryotic MT isoform is well characterized and, as known, this isoform binds exclusively copper differently from MT isoforms from other sources [3]. We found that the column herein utilized was able to efficiently chelate either Cu^{2+} or Ni^{2+}. The same approach was tried in order to test the possibility of purifying the fish MT by loading the column with $CdNO_3{\cdot}4H_2O$, instead of Ni^{2+} as shown in Fig. 1B. In that case, we observed that Cd^{2+} really bound to the column resin, confirmed by Cd^{2+} determination by atomic absorption spectrometry after washing the column with EDTA (result not shown), though the protein could not be recovered during imidazol elution step. Most probably the protein passed through the column did not bind to the Cd^{2+} attached to the resin. At our understanding, MT extracted from the liver of fishes would have quite different affinity for Cd^{2+} and Ni^{2+}, which could explain why it did not bind to the Cd^{2+} attached to the column; however, it did bind to Ni^{2+}, as confirmed by Cd^{2+} and Ni^{2+} determination from MT positive fractions as shown in Fig. 3B. As reported in the literature [19], apoMTs have a non-rigid nature thus a disordered structure and two different cysteine clusters, one at the N-terminal (α-domain) and the other at the C-terminal (β-domain). Each cluster is able to bind four and three metal ions, respectively. Moreover, it was already demonstrated that Cd-MT are able to metal fluxionality provided by Cd-NMR saturation transfer experiments, which established the possibility of metal exchange within the three-metal cluster (β-domain), including inter- and/or intramolecular metal exchange, as well. Most probably due to different affinity for both metals along with those structural singularities attributed to MTs may explain why MT extracted from fishes, presumably metal-loaded in vivo, was able to bind to Ni^{2+}-loaded resin though it did not bind to Cd^{2+}-loaded resin.

In the procedure herein described, two different systems of protein elution from the column were tested: imidazol (up to 500 mM) and EDTA (up to 0.1 mM) continuous gradients. Imidazol is recommended for the elution of recombinant His tag proteins bound to Ni^{2+}-loaded columns. That system was effective toward elution of MT purified from the liver of fishes by utilizing the Ni^{2+}-loaded column. On the other hand, elution with EDTA was efficient for a highly pure separation of the CUP1A metallothionein eluted from the Cu^{2+}-loaded column (Fig. 1A). Usually, EDTA (1 mM) is recommended to remove the metal bound to the resin once protein elution is finished. Nevertheless, MT extracted from yeast cells was eluted by EDTA continuous gradient (Fig. 1A), although same approach did not work as well as imidazol continuous gradient for the purification of the MT isolated from Ni^{2+}-loaded columns. We believe that such difference was due to the kind of metal loading the column and their capability to chelate EDTA.

Affinity chromatography has been a widespread and powerful technique for purification of mainly recombinant proteins modified with specific sequences, e.g., polihistidine tails, enzymes, monoclonal antibodies, DNA-binding proteins and so on. The technique is scarcely described for the isolation of native proteins unless they have any specific structural characteristic that allows a group specific adsorption. On that, metallothioneins have a particular cysteine-rich

structure. An option for their purification is the application of covalent affinity chromatography by thiol–disulphide interchange, as already described in the literature by Kabzinski [20,21]. Although covalent chromatography by thiolated resins may be a useful technique for the isolation of metallothioneins, the entire procedure must be performed with the protein on its completely reduced form, so its application presupposes the isolation of the apoprotein only. Moreover, thiol–disulphide interchange procedure is usually timing-consuming and more prone to failure and to loss of protein mass because of the particularities for sample preparation, deaeration of buffers, elution step and column regeneration as well as the fact that it is a methodology where free catalytic heavy metals must be avoided as much as possible because of the risk of oxidation of either the protein or the resin thiol-groups content.

The method herein described for the purification of metallothioneins through metal-cysteine affinity chromatography by utilizing a metal-chelating resin is a simple as well as a rapid method and, as it is a one-step procedure, the loss of protein mass is greatly reduced during the purification procedure. Another important quality of the method herein proposed is the concentrating effect, intrinsic to affinity chromatography, which enables large volumes to be processed.

An important consideration to be addressed is that the development of a method for the isolation and purification of metallothioneins from fishes or other species found in waters of the Amazon basin is a valuable tool for the study and control of the regional environment.

Acknowledgements

This work has been supported by CNPq (Brazilian National Research Council) and INPA (National Institute for Research in the Amazon). R.T.H., M.D. and A.L.V. were recipients from fellowships from CAPES (Ph.D.), PROSET-CNPq (Regional Development), and CNPq (Research), respectively.

References

[1] M. Margoshes, B.L. Vallee, J. Am. Chem. Soc. 79 (1957) 4813.
[2] J. Hidalgo, M. Aschner, P. Zatta, M. Vasak, Brain Res. Bull. 15 (2001) 133.
[3] P. Coyle, J.C. Philcox, L.C. Carey, A.M. Rofe, Cell Mol. Life Sci. 59 (2002) 627.
[4] M. Sato, M. Kondoh, Tohoku J. Exp. Med. 196 (2002) 9.
[5] K. Nostelbacher, M. Kirchgessner, G.I. Stangl, J. Chromatogr. B: Biomed. Sci. Appl. 744 (2000) 273.
[6] D.C. Simes, M.J. Bebianno, J.J.G. Moura, Aquat. Toxicol. 63 (2003) 307.
[7] C.S. Carvalho, H.S.S. Araújo, M.N. Fernandes, Comp. Biochem. Physiol. B 137 (2004) 225.
[8] R. Verma, S. Chen, R. Feldman, D. Schieltz, J. Yates, J. Dohmen, R.J. Deshaies, Mol. Biol. Cell 11 (2000) 3425.
[9] R. Dallinger, Y. Wang, B. Berger, E.A. Mackay, J.H.R. Kägi, Eur. J. Biochem. 268 (2001) 4126.
[10] M.J. Stillman, W. Cai, A.J. Zelazowski, J. Biol. Chem. 262 (1987) 4538.
[11] D.M. Bollag, S.J. Edelstein, Protein Methods, Wiley-Liss Inc., NY, 1996.
[12] O. Perceval, Y. Couillard, B. Pinel-Alloul, A. Giguere, P.G. Campbell, Aquat. Toxicol. 69 (2004) 327.
[13] R.J. Brown, T.S. Galloway, D. Lowe, M.A. Browne, A. Dissanayake, M.B. Jones, M.H. Depledge, Aquat. Toxicol. 66 (2004) 267.
[14] T.S. Galloway, R.J. Brown, M.A. Browne, A. Dissanayake, D. Lowe, M.B. Jones, M.H. Depledge, Environ. Sci. Technol. 38 (2004) 1723.
[15] B. Berthet, C. Mouneyrac, J.C. Amiard, C. Amiard-Triquet, Y. Berthelot, A. Le Hen, O. Mastain, P.S. Rainbow, B.D. Smith, Arch. Environ. Contam. Toxicol. 45 (2003) 468.
[16] R.W. Olafson, J.A.J. Thompson, Mar. Biol. 28 (1974) 83.
[17] H.L. Ley, M.L. Failla, D.S. Cherry, Comp. Biochem. Physiol. B 74 (1983) 507.
[18] Affinity Chromatography—Principles and Methods, Amersham Pharmacia Biotech, Uppsala, 1999, p. 95 (Chapter 5).
[19] N. Romero-Isart, M. Vasak, J. Inorg. Biochem. 88 (2002) 388.
[20] A.K. Kabzinski, Biomed. Chromatogr. 12 (1998) 281.
[21] A.K. Kabzinski, Biomed. Chromatogr. 14 (2000) 160.