Environ Chem Lett (2017) 15:717–724
DOI 10.1007/s10311-017-0644-0

ORIGINAL PAPER

Identification of protein biomarkers of mercury toxicity in fish

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Received: 19 December 2016 / Accepted: 29 May 2017 / Published online: 7 June 2017

Abstract Bioaccumulative metals such as mercury are found in increasing amounts in fish and their consumers. In the region of the Madeira River, in the Brazilian Amazon, mercury (Hg) is a predominant contaminant in the aquatic ecosystem. There is therefore a need to find specific biomarkers of mercury toxicity in fish to monitor contaminations. Here, mercury-bound proteins were identified in the liver tissues of fishes *Mylossoma duriventre* and *Brachyplatystoma rousseauxii*. Mercury was quantified in liver tissue, pellets and protein spots by graphite furnace atomic absorption. Proteins were fractionated by two-dimensional polyacrylamide gel electrophoresis and identified by mass spectrometry with electrospray ionization. We identified nine proteins linked to mercury and that presented biomarker characteristics of mercury. Among the proteins identified, isoforms of parvalbumin, ubiquitin-40S ribosomal protein S27a, brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 2 and betaine–homocysteine S-methyltransferase 1 are notable for having the molecular function of binding to metallic ions.

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Keywords Mercury in fish · Biomarkers · Metalloproteomics · Mass spectrometry · Proteins · Atomic absorption spectrometry

Introduction

In the aquatic environment, mercury pollution is considered very dangerous for fish and wildlife (Wiklund et al. 2017). In this environment, mercury undergoes methylation, which has been associated with the activity of several anaerobic microorganisms, including sulfate-reducing bacteria, iron-reducing bacteria and methane-producing bacteria (Hellal et al. 2015; Perrot et al. 2015; Zeng et al. 2016). Mercury in its organic form, e.g., methylmercury, is more detrimental to animals, accumulating in the body over time and causing damage to both animals and their consumers (Avramescu et al. 2011). Experiments with fish have shown that mercury affects the brain, muscles, liver and the immune and reproductive systems, in addition to inducing structural cell degeneration, oxidative stress, energy metabolism variations and calcium homeostasis (Inácio 2006; González-Estecha et al. 2014; Macirella et al. 2016). Due to their tendency to bioaccumulate and biomagnify through the food chain, mercury compounds represent substances of great concern to human health (Macirella et al. 2016). Thus, there is a great need to find specific protein biomarkers of mercury toxicity in fish to monitor their levels in regions impacted by anthropogenic activities.

Protein biomarkers are proteins/enzymes that reflect changes in biological responses at different levels, pathological or pharmacological responses to a therapeutic intervention, stress or responses to environmental contaminants such as toxic metals, e.g., mercury, cadmium, arsenic and lead (Senger et al. 2010; Tenório-Daussat et al.

2014). Unlike indicators, protein biomarkers indicate the biological effects of pollutants, which are not seen in uncontaminated environments (Wallace et al. 2016). Therefore, a good protein biomarker must be sensitive to the presence of toxic elements in the environment, aiming to monitor these toxic metals through analysis of these biomarkers (Van Gestel and Van Brummelen 1996; Senger et al. 2010). For this purpose, the species *M. duriventre* and *B. rousseauxii* of Amazon region were chosen because of presenting historical of high concentrations of mercury in their tissues (Braga et al. 2015), occupy different trophic levels, *M. duriventre* being herbivorous (Queiroz et al. 2013) and *B. rousseauxii* carnivorous (Queiroz et al. 2014), and are highly consumed by the riverine population of Amazon region. Considering that higher species in the food chain tend to have higher concentrations of mercury and consequently a higher risk of human exposure to mercury (revised in Rivera et al. 2016), this study seeks to identify biomarkers of mercury exposure in Amazonian fish species to increase the reliability of analysis and to facilitate monitoring of this metal by Brazilian Environmental Agencies.

Materials and methods

Study site and sample collection

The fish species involved in the study, *M. duriventre* and *B. rousseauxii*, were captured from the Madeira River in the area covered by the Jirau Hydroelectric Power Plant, Porto Velho, Rondônia, Brazil. Fish catch points are : a) S 09°16′12.8″, W 064°41′14.1″, and b) S 09° 11′16.98″, W 064° 36′44.53″. Six adult carnivorous *B. rousseauxii* fish averaging 95 cm, mean weight of 25 kg, and ten adult herbivorous *M. duriventre* fish of approximately 20 cm and mean weight of 0.8 kg were captured. From these, hepatic tissue samples were collected, samples pooled and homogenized and their proteins fractionated by two-dimensional electrophoresis and identified by mass spectrometry (Vieira et al. 2015). To quantify total protein, the Biuret method was used, and total mercury was determined by graphite furnace atomic absorption spectrometry after acid mineralization of liver tissue, pellets and protein spots. The methodology was optimized and published by Moraes et al. (2012).

Two-dimensional electrophoresis fractionation

The electrophoretic runs were performed according to our protocols (Braga et al. 2015). Briefly, protein pellets containing proteins with molecular masses less than 90 kDa were solubilized in a solution containing urea, thiourea, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, ampholytes, bromophenol blue and dithiothreitol. The strips were hydrated for 12 h with 1.5 mg mL^{-1} of protein, subjected to isoelectric focusing, alkylated and reduced in equilibrium solutions. The strips were then applied to 15% (m/v) polyacrylamide gels and subjected to two-dimensional electrophoresis. After the run, the proteins were stained with colloidal Coomassie for 72 h and the bleached gels were washed with ultrapure water, digitized and analyzed using ImageMaster Platinum software version 7.0 to obtain spots, matching, isoelectric point and molecular masses of spots.

Identification of proteins by mass spectrometry with electrospray ionization

Protein spots that contained mercury were cut from the gel, and the dye was reduced and alkylated, followed by tryptic digestion of the proteins. The peptides were eluted and analyzed by a nanoACQUITY UPLC-Xevo QT-MS mass spectrometer with an electrospray ionization system (Waters, Manchester, UK)(Braga et al. 2015). Proteins were identified using the Otophysi and UniProt databases (UniProt 2016).

Results and discussion

Determination of total mercury in liver tissue and pellets

The determination of total mercury was carried out directly in the hepatic tissues of *M. duriventre*: 212 ± 4 μg kg^{-1} and *B. rousseauxii*: 582 ± 9 μg kg^{-1} as well as in pellets

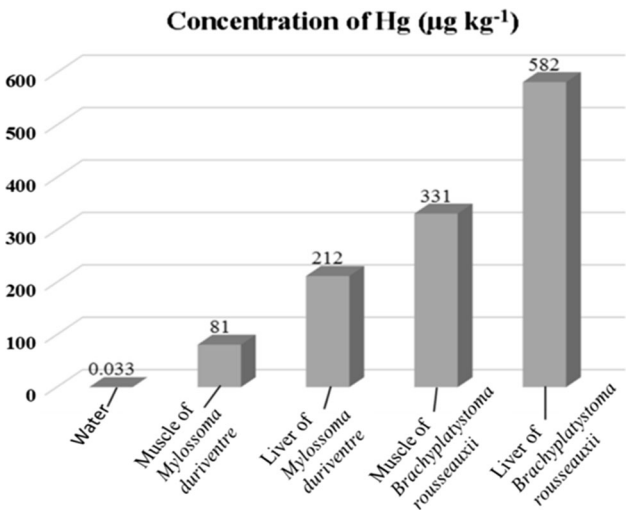

Fig. 1 Results obtained in the determination of mercury (μg kg^{-1}) in the muscular and hepatic tissues of the two fish species studied. The results show higher amounts of mercury in the hepatic tissues of the two specimens, as well as the biomagnification of this metal in *B. rousseauxii*, which is of a trophic level superior to *B. rousseauxii*

of molecular mass lower than 90 kDa and greater than 90 kDa. In pellets greater than 90 kDa, no mercury was detected in any of the tissues. In pellets of lower molecular weight, 138 ± 2 and 379 ± 6 μg kg^{-1} of mercury was detected for *M. duriventre* and *B. rousseauxii* pellets, respectively. The mercury was also quantified in the water of the collection points: 0.033 μg kg^{-1}(Fig. 1). The certified standard DORM 4-NRC (410 ± 55 μg kg^{-1} of Hg) was used to validate the mercury determination method (Moraes et al. 2013), presenting readings of 408 ± 3 μg kg^{-1} Hg, close to the value indicated by the manufacturer. Analyzing the data obtained, more mercury was observed in the hepatic tissue and pellets of *B. rousseauxii*; this was because the liver has a detoxifying function in the organism and, because mercury is a bioaccumulative toxic metal, a greater amount of this bioaccumulation can occur in animals at the top of the chain. In the liver, there is a greater diversity of proteins, including proteins with thiols, hydroxyls and amine groups that have greater affinity for mercury. Then, the amount of mercury was measured in the protein spots obtained in the two-dimensional electrophoresis.

Determination of total mercury in protein spots

For each fish species, two-dimensional electrophoresis runs were performed in triplicate for quantification of mercury in the spots and one run in triplicate to identify the proteins by mass spectrometry. The gels presented reproducibility higher than 75 ± 2% and 218 ± 3 spots for *M. duriventre* and 93 ± 4% and 289 ± 8 spots for *B. rousseauxii* (Fig. 2). The spots obtained by two-dimensional electrophoresis allowed the identification of proteins with higher amounts of mercury atoms attached to them. For this identification, the ratio of the number of mercury atoms per molecule of protein found in the spot was calculated (Moraes et al. 2013). The results showed a relation of up to four mercury atoms for each protein molecule in

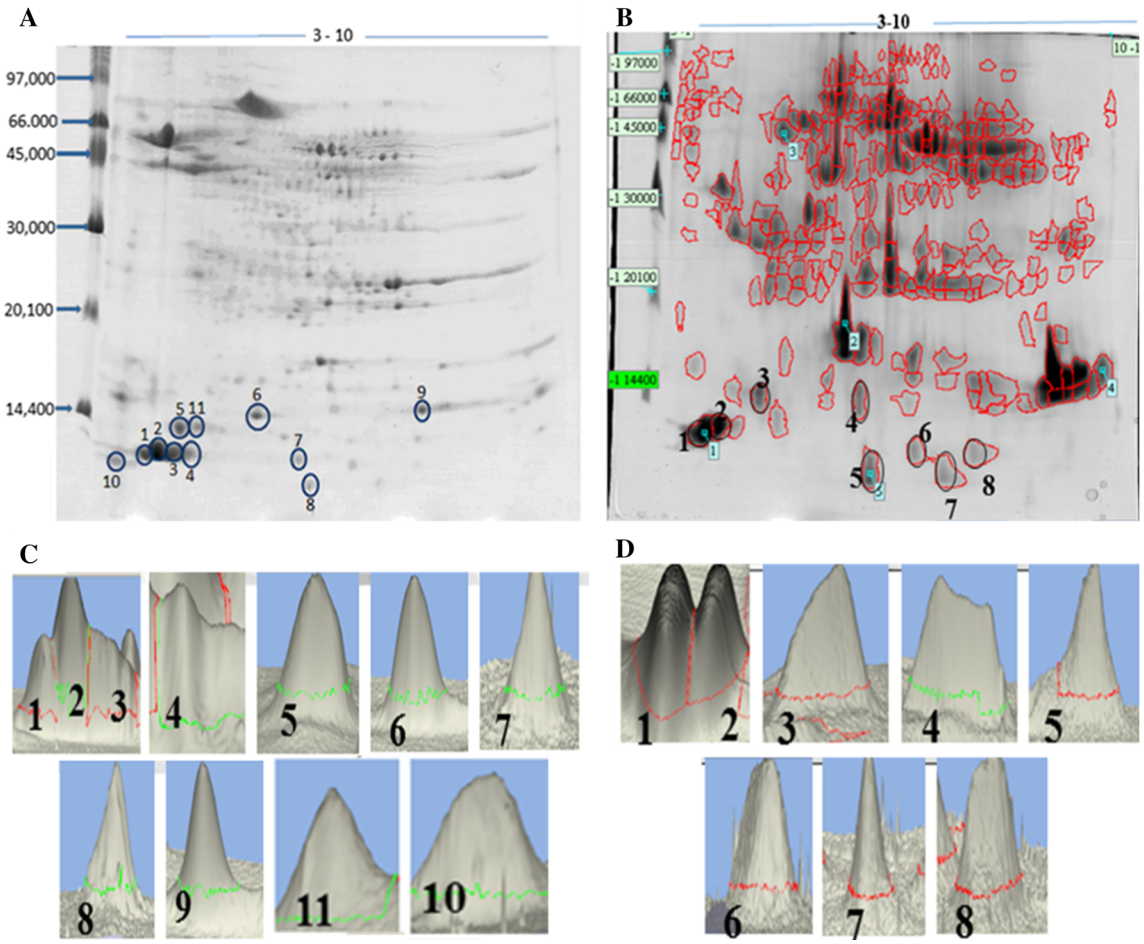

Fig. 2 Representative images of two-dimensional electrophoresis of the liver of *M. duriventre* (**a**) and *B. rousseauxii* (**b**) with spots that showed mercury and had the proteins identified in *M. duriventre* (**c**) and *B. rousseauxii* (**d**). Gel 15% w/v, pH range 3–10 and molecular mass standards of 14–97 kDa from bottom to top in the vertical direction. The images were analyzed by ImageMaster Platinum 7 software to detect the spots. As can be seen in the figures, mercury was found bound only to low molecular weight proteins

the two species studied (Table 1). Studies by our group had already shown this relationship for other species of fish (Moraes et al. 2013; Braga et al. 2015; Vieira et al. 2015). This Hg/protein ratio also depends on the affinity of each protein for the element and/or the amount of mercury found in fish tissues. The protein spots that presented higher levels of mercury were submitted to mass spectrometric analysis for the identification of proteins.

Proteins identified by mass spectrometry with electrospray ionization

Mass spectrometric analysis of protein spots with higher concentrations of mercury revealed nine proteins, fatty acid-binding protein 10-A, interleukin enhancer-binding factor 2 homolog, parvalbumin, mediator of RNA polymerase II transcription subunit 11, 5-hydroxyisourate hydrolase, ubiquitin-40S ribosomal protein S27a, brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 2, betaine–homocysteine S-methyltransferase 1 and Ig heavy chain V region 5A (Table 2). The proteins/enzymes characterized in the study are metalloproteins or metal-binding proteins.

Fatty acid-binding protein 10-A,liver basic (fabp10a) is a protein that exists in several isoforms, is bound to hydrophobic ligands in the cytoplasm and may be involved in the transport of intracellular lipids (Chmurzyńska 2006). The peptide sequences of this protein mainly exhibit arginine, glutamine and leucine residues. However, the literature reports the presence of hydrophobic amino acid residues such as n-acetylmethionine having a thiol group in its structure (Weiss et al. 2002; Chmurzyńska 2006). The presence of thiol groups may justify the incorporation of mercury by fabp10a isoforms, considering the great affinity of thiol groups for mercury (Pearson 1963; Garcia et al. 2006). Fabp10a was identified bound to mercury in spot M4 of *M. duriventre* and spots B1 and B9 of *B. rousseauxii*. In both species, the spots presented significant expression and the data obtained by graphite furnace atomic absorption showed a stoichiometric relation with mercury; it can thus be inferred that this protein can act as a mercury biomarker (Braga et al. 2015).

Interleukin enhancer-binding factor 2 homolog is a protein that acts predominantly as a heterodimeric complex with interleukin enhancer-binding factor 3. This complex regulates the transcription of indeterminate genes (Heinrich et al. 1998). Interleukin enhancer-binding factor 2 homolog

Table 1 Values obtained in the determination of mercury concentration by graphite furnace atomic absorption in the protein spots, molecular mass and pI of the spots and estimation of the number of mercury atoms per protein molecule in *M. duriventre* and *B. rousseauxii* liver tissue samples

Spots Proteins	Molecular mass (kDa)	pI	Protein mass ($\mu g\ g^{-1}$)	Concentration of Hg ($mg\ g^{-1}$)	N° of molecules in protein spot $\times 10^{12}$	N° of atoms Hg $\times 10^{12}$	N° of atoms de Hg by protein molecules (approximately)
Spot M1	12.50	3.50	30.00	16.70	14	15	1.00
Spot M2	12.80	3.80	19.30	13.80	9	8	0.88
Spot M3	14.20	4.40	6.10	32.70	3	6	2.00
Spot M4	14.00	6.00	2.90	34.60	1	3	3.00
Spot M5	10.70	6.20	7.30	31.80	4	7	1.75
Spot M6	11.60	6.90	3.30	60.60	2	6	3.00
Spot M7	10.50	7.40	3.90	68.10	2	8	4.00
Spot M8	11.40	7.90	3.50	38.00	2	4	2.00
Spot B1	12.50	3.60	15.20	13.10	7	6	0.85
Spot B2	12.50	3.70	59.10	14.70	28	26	0.92
Spot B3	12.30	4.00	32.90	15.20	16	15	0.93
Spot B4	12.30	4.30	7.60	13.10	4	3	0.75
Spot B5	13.40	4.20	11.30	14.70	5	5	1.00
Spot B6	14.20	5.30	6.10	16.40	3	3	1.00
Spot B7	12.40	6.10	2.10	31.80	1	2	2.00
Spot B8	11.30	6.20	2.00	66.80	1	4	4.00
Spot B9	14.70	8.00	5.70	11.70	2	2	1.00

The results show a strong relation between the amount of mercury atoms and protein molecules identified in the studied species

Spot M spot of liver from *M. duriventre*, *Spot B* spot of liver from *B. rousseauxii*, *pI* isoelectric point. Results expressed in $mg\ g^{-1}$

Table 2 Proteins identified using mass spectrometry with electrospray ionization in *B. rousseauxii* and *M. duriventre* hepatic tissue samples

Spot Gel	Proteins	Access	*Score*	pI/Mm of *spot*	MmESI	Mm theoretical Uniprot	Cover (%)	Molecular function
Spot B1	Fatty acid-binding protein 10-A. liver basic	FA10A_DANRE	96.9797	3.60/12.50	14.90	14.00	7.94	Bile acid binding, fatty acid binding, transporter activity
Spot B2	Interleukin enhancer-binding factor 2 homolog	ILF2_DANRE	16.5826	3.70/12.50	44.10	42.90	6.98	Activator, immune response, positive regulation of transcription, DNA templated, transcription, DNA templated
Spot B3	Parvalbumin beta	PRVB_SQUCE	73.639	3.00/12.30	11.979	11.263	9.43	Calcium ion binding, metal binding
Spot B6	Mediator of RNA polymerase II transcription subunit 11	MED11_DANRE	164.0934	5.30/14.20	13.231	12.786	21.93	RNA polymerase II transcription cofactor activity, activator
Spot B7	5-hydroxyisourate hydrolase	Q06S87	70.7215	6.10/12.40	15.80	15.50	5.07	Hydrolase activity, acting on carbon–nitrogen (but not peptide) bonds, in linear amides, hydroxyisourate hydrolase activity
Spot B8	Ubiquitin-40S ribosomal protein S27a	RS27A_ICTPU	71.5344	6.20/11.30	19.80	17.90	5.77	Structural constituent of ribosome, metal binding
Spot B9	Fatty acid-binding protein 10-A. liver basic	FA10A_DANRE	210.8158	8.00/14.70	14.90	14.00	23.02	Bile acid binding, fatty acid binding, transporter activity
Spot M1	Parvalbumin-2	PRV2_DANRE	1790.152	3.50/12.50	12.20	11.60	10.09	Calcium ion binding, metal binding
Spot M2	Parvalbumin alpha	PRVA_CYPCA	311.8534	3.6/12.80	11.816	11.451	17.43	Calcium ion binding, metal binding
	Parvalbumin-2	PRV2_DANRE	311.8534		12.253	11.622	17.43	Calcium ion binding, metal binding
	Parvalbumin beta	PRVB_SQUCE	197.3065		11.979	11.263	16.98	Calcium ion binding, metal binding
Spot M3	Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 2	BI2L2_DANRE	23.3293	4.40/14.20	58.70	57.60	3.29	Cytoskeletal adaptor activity, zinc ion binding
Spot M4	Fatty acid-binding protein 10-A. liver basic	FA10A_DANRE	96.9797	6.00/14.00	14.80	14.00	7.94	Bile acid binding, fatty acid binding, transporter activity
Spot M5	Ubiquitin-40S ribosomal protein S27a	RS27A_ICTPU	983.4811	6.20/10.70	19.80	11.50	11.54	Structural constituent of ribosome, metal binding
Spot M6	Betaine–homocysteine S-methyltransferase 1	BHMT1_DANRE	2045.392	6.90/11.60	46.50	44.10	5.25	Cytoskeletal adaptor activity, zinc ion binding
Spot M7	Ig heavy chain V region 5A	HV05_CARAU	84.9197	7.40/10.50	13.30	12.80	19.83	Antigen binding
Spot M8	Betaine–homocysteine S-methyltransferase 1	BHMT1_DANRE	3020.698	7.90/11.40	46.449	44.066	14.75	Cytoskeletal adaptor activity, zinc ion binding

We identified seven mercury-bound proteins with biomarker characteristics of this metal in fish

Spot B spot of liver from *B. rousseauxii*, *Spot M* spot of liver from *M. duriventre*, *pI* isoelectric point, *Mn* molecular mass, *ESI* electrospray ionization mass spectrometry

exhibits peptide sequences formed primarily by leucine residues, tyrosine and also peptide sequences of chemokines, cytokines showing cysteine residues (Heinrich et al. 1998). The incorporation of mercury in the structure of interleukin enhancer-binding factor 2 homolog can be associated with the presence of chemokines that have cysteine residues, amino acids that present/display a thiol group which has the characteristics of a weak base and has great affinity for mercury (Pearson 1963; Garcia et al. 2006). Spot B2 from hepatic tissue of *B. rousseauxii* was characterized as being this protein with mercury incorporated in its structure. As this spot showed a large salience in the gel and the data obtained by graphite furnace atomic absorption showed that there is a stoichiometric relation with mercury, it can be inferred that this protein can act as a mercury biomarker.

Parvalbumin is a calcium-binding, metal-binding metalloprotein under normal conditions and is involved in muscle contraction and relaxation (Carvalho et al. 2014; Freidl et al. 2016). This protein can perfectly associate with mercury, as it is also a metal and has characteristics capable of dislocating the calcium atoms and competing for its active sites. In *M. duriventre* and *B. rousseauxii* parvalbumin and its isoforms were found with a stoichiometric correlation of approximately one atom of mercury per protein molecule, the same relation found by other studies with muscular and hepatic tissues of *Cichla* sp.(Vieira et al. 2015) and muscle of *B. rousseauxii* (Braga et al. 2015). This ratio of mercury atoms to protein molecules has often increased as the concentration of mercury increases in fish tissues. In studies with *B. rousseauxii*, for example, which presented greater mercury in tissues than *M. duriventre*, the atom/molecule ratio was also increased. Considering these characteristics and its history as a mercury-associated protein in Amazonian fish, parvalbumin can be considered a potential biomarker of this toxic element in the tissues.

Mediator of RNA polymerase II transcription subunit 11 is the enzyme component of the mediator complex and coactivator of the regulated transcription of RNA polymerase II-dependent genes. Its main function is to mediate the transmission of information from regulatory proteins specific to basal RNA polymerase II, which acts as transcription machinery (Kim et al. 1994). This enzyme has a peptide sequence formed mainly by alanine, histidine and leucine, lacking amino acids with thiol groups (Kim et al. 1994). The literature does not present any work that discusses the possibility of mercury binding to mediator of RNA polymerase II transcription subunit 11. However, because this enzyme has available divalent metal-binding sites, it can form bonds with Hg^{2+} ions. Thus, it can be inferred that mercury may be incorporated into this protein by non-specific bonds forming a metal-binding protein, not being configured as a metal cofactor (Garcia et al. 2006). Spot B6 from hepatic tissue of *B. rousseauxii* was identified as being this enzyme with mercury incorporated in its structure. As this spot showed significant expression and the data obtained by graphite furnace atomic absorption demonstrated that there is a stoichiometric relation with mercury, it can be inferred that this protein can act as a mercury biomarker, even though it does not present specific groups for the binding of this element.

5-Hydroxyisourate hydrolase, an enzyme that belongs to the hydrolase family, participates in reactions involving peptide bonds and carbon–nitrogen bonds, specifically in the cyclic amides of other hydrolases. This enzyme participates in the metabolism of purines and the degradation of uric acid and acts as a catalyst in the hydrolysis of 5-hydroxystearate to 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (Lee et al. 2005). In the literature, no studies have been found to discuss the possibility of mercury binding to 5-hydroxyisourate hydrolase. However, because this enzyme has available divalent metal bonding sites, it can also form bonds with ions such as Hg^{2+} (Lee et al. 2005; Garcia et al. 2006). These non-specific bonds characterize a metal-binding protein, not configuring mercury, in this case, as a metal cofactor (Garcia et al. 2006). Spot B7 from hepatic tissue of *B. rousseauxii* was identified as being this enzyme with mercury incorporated in its structure. However, this spot did not present significant expression in the gel, even considering that the data obtained by graphite furnace atomic absorption showed a stoichiometric relation with mercury; therefore, more detailed studies on its profile as a possible biomarker will be necessary.

Ubiquitin-40S ribosomal protein S27a is a component of the 40S subunit of the ribosome; it can be found in the cellular cytoplasm linked to other proteins performing different functions such as DNA repair, endoplasmic reticulum, lysosome and protein degradation, cell cycle regulation, kinase modification and activation processes of NF-kappa-B (Karsi et al. 2002; UniProt Consortium 2016). Ubiquitin is a metal-binding protein that binds to zinc, which is in the same family as mercury. This similarity may explain the fact that mercury is associated with ubiquitin, because it has higher affinity for the protein than zinc itself (Garcia et al. 2006). Both *M. duriventre* and *B. rousseauxii* showed mercury bound to ubiquitin obeying a ratio of two and four mercury atoms per molecule of protein, respectively. Studies with muscular and hepatic tissues of *Cichla* sp. have already shown a relation of up to one mercury atom per ubiquitin molecule (Vieira et al. 2015). This ratio increases to two mercury atoms per molecule of protein in *B. rousseauxii* muscle tissue (Braga et al. 2015). Considering the frequency of ubiquitin binding to mercury, it may be considered a promising biomarker.

Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 2 is a protein that induces the formation of structures of flat or small curved membranes and may present more than one isoform (UniProt 2016). It can act as an adapter protein, aiding in the formation of actin bundles and in the reorganization of the actin cytoskeleton in response to bacterial infections (Scita et al. 2008). The literature does not present any work that discusses the possibility of mercury binding to brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 2. However, this protein presents in its peptide sequence mainly arginine and serine residues with carboxylic and terminal amino groups, which have binding sites for divalent metals, so it may form bonds with ions such as Hg^{2+} (Scita et al. 2008). These bonds, although not specific, characterize a metal-binding protein (Garcia et al. 2006). Spot M3 of *M. duriventre* hepatic tissue was identified as being this protein with mercury incorporated in its structure. This spot presented good resolution in the gel and the data obtained by graphite furnace atomic absorption demonstrated that there is a stoichiometric relation with mercury, which qualifies it as a biomarker candidate.

Betaine–homocysteine S-methyltransferase 1 is a zinc metalloenzyme involved in the regulation of homocysteine metabolism and presents more than one isoform (UniProt 2016). It is found exclusively in the liver and kidney of animals and humans. It converts betaine and dimethylglycine homocysteine to methionine and also participates in the irreversible oxidation reaction of choline (González et al. 2004; UniProt 2016). Betaine–homocysteine S-methyltransferase 1, due to the presence of zinc bound to the coordination sites formed by thiol groups in the peptide sequence, may have Hg^{2+} ions incorporated at these coordination sites (Pearson 1963; González et al. 2004; Garcia et al. 2006). Spots M6 and M8 from *M. duriventre* were identified as being this metalloenzyme with mercury incorporated in its structure.

Ig heavy chain V region 5A is a protein that constitutes the large immunoglobulin polypeptide subunit (UniProt 2016). It is a typical antibody consisting of two heavy chains and two immunoglobulins (Ig) (Hsu 1994). The heavy chains contain a number of immunoglobulin domains, usually with a variable domain (VH) which is important for antigen binding, and several constant domains, CH_1 and CH_2 (Hsu 1994). The immunoglobulin monomers are formed of four peptide chains linked by disulfide bonds of cysteine thiol groups (Hsu 1994). The presence of cysteine in Ig heavy chain V region 5 may favor the binding of Hg^{2+} ions (Pearson 1963; Garcia et al. 2006). Spot M7 of *M. duriventre* hepatic tissue was identified as being this protein with mercury incorporated in its structure. This spot showed good resolution in the gels and the data obtained by graphite furnace atomic absorption showed a stoichiometric relation with mercury (Table 1), which qualifies it as a mercury biomarker candidate.

Conclusion

The methodology described here can be applied in the identification of biomarkers of mercury exposure in fish because the mercury-associated proteins identified play an important role in fish physiology. As mentioned in this study, parvalbumin and ubiquitin were the most promising proteins to become biomarkers of mercury in fish, not only because of the amount of mercury attached to them, but also because of the frequency of mercury binding to these proteins in the liver of different species of Amazonian fish. Thus, the biomarkers of mercury exposure characterized in the present work will contribute to the monitoring programs of organisms exposed to this metal in the Amazon region developed by Brazilian Environmental Agencies.

Acknowledgements The authors thank the Brazilian research funding agencies FAPESP (processes 2013/21297-1 and 2014/02668-1) and ANEEL/ESBR (P&D: 6631-0001/2012/Contract Jirau 004/201) for their financial support.

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