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Full Length Article

Identification of Some Novel Biomarkers for Assessment of Copper Sulphate Toxicity in Liver of *Puntius javanicus* through Proteomic Analyses

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Abstract

Copper is extensively released into the environment, affecting the natural habitat of surrounding organisms especially the aquatic life. The aim of this study is to explore the different protein expressions on different concentrations of $CuSO_4$. *Puntius javanicus* liver was used as a tool to investigate the biomarkers and mechanisms of copper toxicity to *P. javanicus* through proteomic assessment after being exposed with different concentrations of $CuSO_4$ (0, 0.1, 0.3, 0.5, 1.0 and 5.0 mg/L). *P. javanicus* liver was extracted (TCA-acetone extraction) and electrophoresed (IEF and 2D PAGE). Ten protein spots were found to be significantly different in their expression and subsequently identified *via* matrix assisted laser desorption/ionization tandem time-of-flight (MALDITOF). Three protein spots were further identified as new alternative biomarkers for copper toxicity namely, zinc finger C4H2 domain-containing protein, insulin gene enhancer protein isl-2a and synaptic vesicle membrane protein VAT-1 homolog-like. The present study could provide an additional understanding on the hepatotoxic mechanisms of copper as well as enhance the development of biomarkers for an efficient monitoring of the contamination level in the environment based on proteomic assessment.

Keywords: Proteomic; Copper; Liver; Biomarker; *Puntius javanicus*; Proteins

Introduction

Copper (Cu) is an element that has many well-known functions. For instance, Cu either in the form of metal ions (Cu^{2+}) or salts ($CuSO_4$ and $CuCl_2$) are considered as vital components in the production of pesticides and fertilizers. The solid form of Cu is also an important component in electrical and electronic devices (Oliveira-Filho *et al.* 2004; Salama 2005; Shomar 2006; Yang *et al.* 2011; Obrador *et al.* 2013). The agricultural application of pesticides and fertilizers had enhanced farm productivity and reduced losses in plantations, resulting in the increase of crop production and quality. However, misapplication and poor management associated with their excessive use may lead to environmental contamination, especially in water bodies as the final target, thus affecting organisms in aquatic ecosystem such as planktons, fish and aquatic plants (Ahmad *et al.* 2016; Basirun *et al.* 2019a).

Cu is an essential heavy metal together with iron, zinc and magnesium, which play a major role as a biological regulator in fish, especially for neurotransmitter function, respiration, reactive oxygen species (ROS) scavenger and as cofactors for numerous enzymes (Lauer *et al.* 2012; Singh *et al.* 2012; Zubair *et al.* 2013). Unfortunately, Cu may become toxic at higher concentrations if it exceeds the internal needs. The toxicity of copper has been described to alter fish behavior from the biomolecular to the cellular level. Simpson *et al.* (2006) discussed the induction of oxidative stress by Cu, which causes damage on cell membranes and other biochemical compounds. This activity has been proven by Varanka *et al.* (2001) and Sabullah *et al.* (2014a) in their studies where the acute effects of copper sulphate ($CuSO_4$) toxicity caused parenchymal abnormalities and nucleus fragmentation in the liver of fish. Cu toxicity also affects both *in vivo* and *in vitro* activity of antioxidants and detoxifying enzymes in fish liver such as superoxide dismutase, catalase, glutathione S-transferase, glutathione peroxidase, metallothionine and

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butyrylcholinesterase (Vieira *et al.* 2009; Chen and Chan 2011; Sabullah *et al.* 2014b). Behavior alteration was also observed in the form of unsteady swimming pattern, decreased sensitivity of avoidance behavior and food consumption associated with sublethal concentration of Cu (Ezeonyejiaku *et al.* 2011; Howcroft *et al.* 2011; Tilton *et al.* 2011; Padrilah *et al.* 2017; Basirun *et al.* 2019b; Fadzil *et al.* 2019).

Liver is one of the most critical organs that can be studied to elucidate the level of stress of an organism caused by environmental factors such as pH, temperature and pollution (Apraiz *et al.* 2006; Wei *et al.* 2008; Jiang *et al.* 2014; Sabullah *et al.* 2017). Moreover, the liver plays a major role in metabolism and storage as this organ may accumulate various xenobiotics for neutralization and detoxification before being distributed to other organs via blood circulation. Dose dependent and time course effects may affect the health status and decreased yield associated with poor quality of fish products. Thus, studies on protein expression in fish liver will help to reveal a significant part of the metabolic activities throughout normal and stressed protein expression or under mixed elective conditions. Toxicoproteomic identification on various types of toxicants was implemented involving the analysis on unique protein expression species (PES) affected by heavy metals, organic compounds and pesticides (Huang and Huang 2011; Jeon *et al.* 2011; Park and Lee 2014).

Puntius javanicus was used in this study as this fish has a commercial value in South East Asia, especially Malaysia (Tacon and Silva 1997). *P. javanicus* was tested to determine the acute effect of $CuSO_4$ on its liver through proteomic analysis based on 2-DE to obtain a hepatocyte protein expression profile map as a database for future research reference. Peptide sequences were determined by MALDITOF-TOF mass spectrometry and used to identify 10 proteins using the Mascot search engine on various types of protein databases due to the unavailability of a proteome database for *P. javanicus* peptides.

Materials and Methods

Specimen treatment and sample preparation

Live adults of *P. Javanicus* (average weight 480 g) were obtained from the Agriculture Development Centre, Bukit Tinggi, Pahang, Malaysia. Fish were divided into six groups (1 group contained nine fish) and acclimatized for 15 days (12 h daylight: 12 h in the dark) in dechlorinated tap water (80 L, pH 6.8 to 7.5, 25 to 27°C) with a fully aerated system. Water was changed once a week with several additions of 5 mL of commercial antifungal solution (contains conditioning salt and anti-white spot) for each change to maintain the cleanliness of the water and to prevent disease. At the end of the acclimatization period, five groups of fish were separately exposed to $CuSO_4$ at the final concentrations of 0.1, 0.3, 0.5, 1.0 and 5.0 mg/L, while one group served as the untreated control. After 96 h of treatment, liver tissues from the control and exposed fish were homogenized under flowing liquid nitrogen to a fine powder. The homogenates were then processed by mixing with acetone at the ratio of 1:10 (w/v) and vortexed for three minutes at room temperature before being centrifuged at 12,000 x g for 10 min. The pellet was recovered and mixed again with acetone and this cleaning step was repeated three times. The extractant was mixed with acetone containing 30% trichloroacetic acid (TCA) at the ratio of 1:10 (w/v), vortexed for three minutes and overnight incubation at -25°C. Then, the mixture was centrifuged at 12,000 × *g* for 10 min. The proteins were washed with chilled acetone twice and followed by mixing with acetone containing 2% dithiotreitol (DTT). The mixtures were then gently vortexed for one minute and then incubated for two hours before being centrifuged at 10,000 × *g* for 10 min. The resulting protein pellets were air-dried and the dried pellets were dissolved in a rehydration buffer (RB; 7 M urea, 2 M thiourea and 4% CHAPS) without any addition of protein carrier and reducing agent. The protein samples were gently vortexed for a minute and centrifuged at 10,000 × *g* for 10 min. The supernatants were recovered followed by determination of protein concentration based on the method by Bradford (1976).

Performing isoelectrofocusing (IEF) and 2D-PAGE

700 μg of protein was added with RB containing freshly prepared 0.2% protein carrier (biolyte 3-10) and 20 m*M* DTT and the volume was adjusted to an end volume of 300 μL. Immobiline strips (17 cm, pH 3–10) were placed onto the samples with mineral oil layered on top to fully cover the samples. IEF was performed on a PROTEAN IEF cell, starting with passive rehydration for 12 h at room temperature, followed by rapid-gradient voltage at 250 V for 15 min, a linear-gradient voltage setting at 10,000 V for three hours and lastly at 60,000 V/h with rapid-gradient voltage. Equilibration of focused strip for reduction (50 m*M* Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.02% bromophenol blue, 65 m*M* DTT) and alkylation (50 m*M* Tris-HCl pH 8.8, 6 *M* urea, 30% glycerol, 2% SDS, 0.02% bromophenol blue, 135 m*M* iodoacetamide) was performed for 15 min in each step at room temperature. Equilibrated strips were placed onto 12.5% SDS-PAGE slab gel, overlaid with a 0.5% agarose sealing solution and electrophoresed at two stages; 1) 16 mA for 30 min and 2) 24 mA for 2.5 h or until the bromophenol blue completely migrated out from the gel. Afterwards, the gels were washed twice with ultrapure water to remove the remaining SDS that could possibly interfere with the staining process later on. The gel was stained with silver nitrate and colloidal Commasie brilliant blue (CBB)

according the preparation method by Yan *et al.* (2000) and Dyballa and Metzger (2009), respectively.

Gel imaging and analysis

Each gel was scanned to obtain its image using calibrated densitometer G-800 (BioRad). Scanning was performed under the transmission mode followed by the type of staining condition, either CBB or silver stain. Then, the image was converted to TIFF format using Quantity One (BioRad) software where the image resolution was changed to 300 dpi and 16-bit depth. Grayscale of the gel image was imported and analyzed using Progenesis Samespots (NonLinear Dynamics) software. A number of protein spots were automatically detected after the process of image alignment, filtering and normalization. Significantly decreased or increased protein expressions were determined using one-way ANOVA followed by classifying *P* value ($P < 0.05$), then spot filtration was carried out by only selecting proteins with a fold change of more than 2.0. Finally, the 10 most significant spots were selected and manually excised from the CBB stained gel and stored at 4°C until subsequent use.

Spot identification

The 10 selected spots that showed significant expression ($P < 0.05$) and fold change greater than 2.0) with variable isoelectric point (pI) and molecular weight (Mw), that were also detected by the CBB stain were sent to the Proteomics International, Institute of Medical Research, QEII Medical Centre, Nedlands, Perth, Australia for analysis. There, the protein spots were destained, trypsin digested and peptide extracted according to the standard method of Bringans *et al*. (2008). Peptides were analyzed by MALDI-TOF/TOF mass spectrometer, which was performed on a 5800 Proteomics Analyzer (AB Sciex). All *de novo*-derived sequences from MS spectra were submitted for Mascot sequence matching software [Matrix Science: https://sysbio-mascot.wehi.edu.au/mascot] starting with database from Swiss-Prot, followed by UniProtKB and NCBInr. The search parameters for the Mascot search engine were as in Table 1.

Results

2D PAGE analysis

No mortality was observed until near the end of the $CuSO_4$ treatment. Samples of *P. javanicus* liver from control and Cu-treated fish were subjected to 2D analysis to determine the significant expression of proteins. The liver protein profile (pI 3 to 10) from control and the highest concentration treatment (5.0 mg/L) stained with silver nitrate are displayed in Fig. 1 with the total number of spots varying from 1600 to 1800 for each treatment. The liver proteomes of control and treated fish were matched and compared and only significantly expressed proteins ($P < 0.05$) and having a fold change of more than 2.0 was considered. Ten labelled protein spots (six upregulated and four downregulated) affected by $CuSO_4$ concentrations were selected for subsequent MS/MS identification.

Table 1: Parameters for the Mascot search engine

Taxonomy	:	Bony vertebrates
Peptide tolerance	:	± 0.4
MS/MS tolerance	:	± 0.4
Peptide charge	:	+1
Mass	:	Monoisotopic
Enzyme	:	Trypsin
Miss cleavage	:	1
Fixed modification	:	Carbamidomethyl

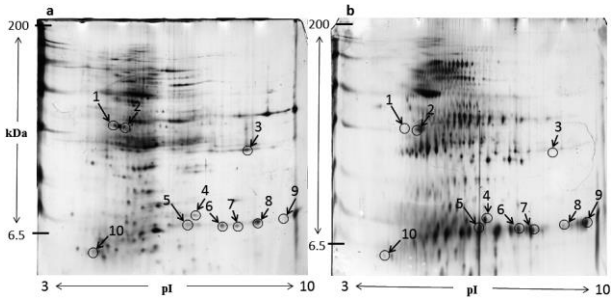

Fig. 1: Representative 2-DE gel image indicating both gels resolved with *P. javanicus* liver proteome which changed in volume after treatment with different concentrations of $CuSO_4$. Protein spots were visualised by silver staining. Arrows indicate the proteins with spot ID that were significantly changed after $CuSO_4$ treatment. (**A**) Control and (**B**) 5.0 mg/L

Protein identification

The Mascot search results are summarized in Table 2. Identification by Mascot search showed that all portions of the MS/MS spectra were similar to proteins from fish species such as *Tribolodon hakonensis, Danio rerio, Salmo salar, Takifugu rubripes, Carassius auratus* and *Cyprinus carpio* as these species have been extensively studied with plentiful data in the public domain. Trypsin (Spot ID 1), zinc finger C4H2 domain-containing protein (ZC4H2) (Spot ID 2), insulin gene enhancer protein isl-2a (islet2a) (Spot ID 3) and haemoglobin subunit beta-A/B (Spot ID 8) showed a decrease ($P < 0.05$) in expression (Fig. 2). Normalized volume on islet-2A and haemoglobin subunit beta A/B were significantly increased ($p < 0.05$) at 0.1 mg/L treatment and downregulated through the concentration treatment. Both proteins could have a role in homeostasis or adaptation effect. Gastrotropin (Spot ID 4), synaptic vesicle membrane protein VAT-1 homolog-like (VAT-1L) (Spot ID 5), haemoglobin subunit beta (Spot ID 6), and haemoglobin subunit alpha (Spot ID 7 and 9) exhibited upregulation associated with the increased concentration of $CuSO_4$. Spot number 10, which was identified as parvalbumin, showed a

Table 2: Differentially expressed proteins in the *P. javanicus* liver proteome after $CuSO_4$ treatment

Spot ID	Peptide sequence hit	Accesion (Species) No.	Identified proteins	Related species	Protein Score	pI (The/Exp)	Mw (The/Exp)	Subcellular location	Regulation
1	NHPGVYGK	B3Y604	Trypsin	*Tribolodon hakonensis*	26	5.61/4.76	25.91/32.30	Extracellular space	↓
2	HLREYK	ZC4H2_DANRE	Zinc finger C_4H_2 domain-containing protein	*Danio rerio*	19	7.06/5.01	26.21/31.76	Cytoplasm	↓
3	QPPHRNHVHK	ISL2A_DANRE	Insulin gene enhancer protein isl-2a	*Danio rerio*	11	8.26/8.34	40.22/26.34	Nucleus	↓
4	K.YHHTSEISGGK.L	B5XB48	Gastrotropin	*Salmo salar*	56	6.74/6.92	14.37/11.833	Cytoplasm	↑
5	AMPELQDGEVKIR	gi410913183 XP003970068.1	PREDICTED: Synaptic vesicle membrane protein VAT-1 homolog-like	*Takifugu rubripes*	45	5.07/6.74	45.60/8.091	Nucleus	↑
6	-.VEWTDAER.S K.LNPDELGPQALAR.C	HBB_CARAU	Hemoglobin subunit beta	*Carassius auratus*	89	7.85/7.67	16.43/9.190	Mitochondria	↑
7	K.ADDIGAEALGR.M	HBA_CYPCA	Hemoglobin subunit alpha	*Cyprinus carpio*	39	8.78/8.1	15.44/9.188	Cellular component	↑
8	-.VEWTDAER.S K.LNPDELGPEALAR.C R.CLIVYPWTQR.F	HBB_CYPCA	Hemoglobin subunit beta-A/B	*Cyprinus carpio*	78	7.89/8.64	16.42/10.231	Mitochondria	↓
9	K.ADDIGAEALGR.M K.LRVDPANFK.I	HBA_CYPCA	Hemoglobin subunit alpha	*Cyprinus carpio*	105	8.78/9.38	15.44/11.438	Cellular component	↑
10	K.LFLQNFSAGAR.A	PRVA_CYPCA	Parvalbumin	*Cyprinus carpio*	36	4.43/4.19	11.50/3.647	Unknown	↑*

* Marked as significantly different with control at 0.5 mg/L $CuSO_4$ treatment and the intensities were lowered at 1.0 and 5.0 mg/L

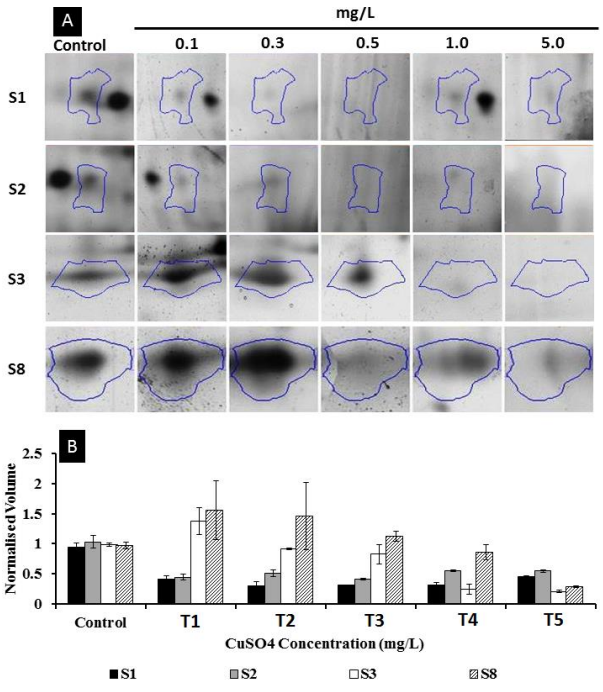

Fig. 2: Downregulated proteins affected by $CuSO_4$ at different concentrations of treatment. S1, S2, S3, and S4 represent trypsin, ZC4H2, Islet-2A and hemoglobin-β in Figure **A**, while T1, T2, T3, T4 and T5 represent the treatment concentrations of 0.1, 0.3, 0.5, 1.0 and 5.0 mg/L in Figure **B**, respectively

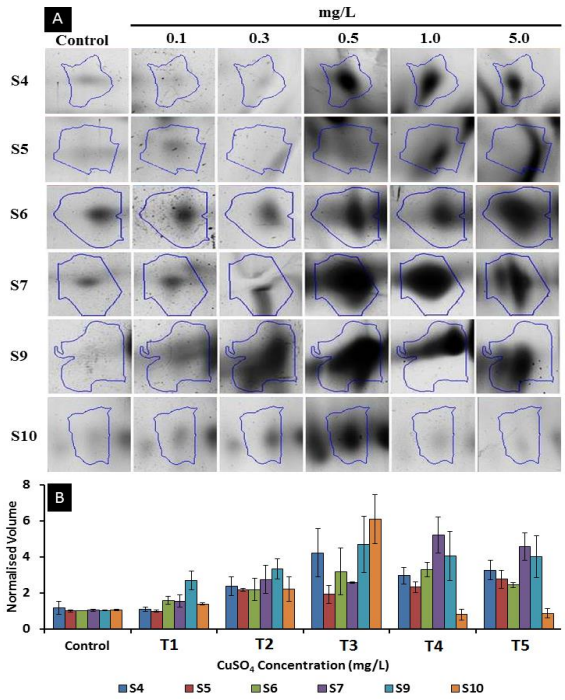

Fig. 3: Upregulation of proteins affected by $CuSO_4$ at different concentrations of treatment. S4, S5, S6 and S10 represent gastrotropin, VAT-1L, haemoglobin-β and parvalbumin, while S7 and S9 shares the same identity as hemoglobin-α in Figure **A**, while T1, T2, T3, T4 and T5 represent the treatment concentrations of 0.1, 0.3, 0.5, 1.0 and 5.0 mg/L, respectively in Figure **B**

sudden upregulation ($P < 0.05$) at 0.5 mg/L $CuSO_4$, but the expression was significantly decreased ($P < 0.05$) at 1.0 and 5.0 mg/L $CuSO_4$ concentrations (Fig. 3).

From the data obtained, the molecular weights of Islet-2A and VAT-1L at 26.34 and 8.10 kDa were much lower compared to the theoretical values which are 40.22 and 45.60 kDa and with the protein scores of 11 and 45, respectively. All of the identified spots showed protein scores of more than 11. The deviation of pI values and molecular weights between apparent and expected result

could be due to different instruments used for the mass spectrometry analysis. Spot ID 7 and 9 shared a same identity from the same species, whereas Spot ID 6 and 8 have the same protein identity but were different in pI and expression profile. This situation where a protein is presented at more than one location in 2D gel is normal.

Discussion

Proteomics is an alternative, efficient and sensitive method to identify proteins affected by environmental pollutants (Breitholtz *et al.* 2006; Marsillach *et al.* 2013). It may aid in answering biological questions as well as understanding the biomolecular mechanisms underlying Cu-induced responses and adaptation in *P. javanicus* liver. Cu toxicity determination based on the proteomic approach has been implemented by previous studies (Simpson *et al.* 2006; Zhang *et al.* 2009; Contreras *et al.* 2010; Chen and Chan 2011, 2012). All of these studies proved that Cu has a toxic effect by altering the regulation of protein related to antioxidants and detoxifying enzymes. In this study, controlled and Cu-treated *P. javanicus* liver were resolved in 2D gels to show differential protein expression maps. Through software analysis based on the changes of normalized values, 10 spots that were significantly affected by Cu were excised and subsequently identified by MS.

Trypsin is a type of protease functioning as a digestive enzyme with the capability to hydrolyze peptide at the carboxyl site chain of arginine and lysine (Cao *et al.* 2000). A sublethal exposure of cadmium caused the overexpression of trypsin in the liver proteome of *Plecoglossus altivelis* and *S. Senegalensis* (Costa 2011; Lu *et al.* 2012). Upregulation of trypsin has been reported to trigger a deleterious effect through the increased generation of ROS and apoptotic compounds (Williams and Henkart 1994; Murn *et al.* 2004; Lim *et al.* 2006). However, the present study showed a downregulation of trypsin in *P. javanicus* liver proteome associated with $CuSO_4$ treatment. This situation is maybe due to the inhibition of this enzyme through the binding of Cu with the side chain of amino acids including the sulhydryl group, and then altering the protein structure by cleaving the disulphide bond (Chen *et al.* 2005).

ZC4H2 and Islet-2A were involved in neurological activity including specification or differentiation outcome (Sun *et al.* 2008; Lombard *et al.* 2011; Hirata *et al.* 2013; Qu *et al.* 2014). The present study found that $CuSO_4$ treatment had caused the downregulation of both of these proteins which continued to decrease as the concentration of $CuSO_4$ treatment increased. There were no specific reports of ZC4H2 and Islet-2A being affected by any type of toxicants, thus, this study shows the novelty of both proteins as an alternative biomarker for toxicants, especially for Cu exposure. These proteins are of zinc finger (ZF) protein type and perhaps their expression behaviors are similar with those reported by Song *et al.* (2008) and Chen and Chan (2012), where the ZF protein in mammalian cells and tilapia fish were downregulated after being exposed to sublethal concentrations of Cu. ZF protein in small-cell lung cancer-derived cell lines and human lung epithelial cells were also downregulated after being exposed to cadmium (Aiba *et al.* 2008; Choi *et al.* 2009). Alteration of ZF protein expression was due to Cu at toxic concentrations being able to replace zinc in the ZF protein, causing dislocation of folding, inactivation as well as degradation of protein as mentioned by Hartwig (2001) on the displacement of zinc in ZF protein by cadmium and cobalt ion.

Gastrotropin, also known as fatty acid binding protein, is involved in lipid uptake and transportation and cholesterol homeostasis for bile production (Besnard *et al.* 2004; Praslickova *et al.* 2012). This protein showed upregulation in expression after being exposed with $CuSO_4$. However, Cu deficiencies caused downregulation of gastrotropin on rat intestine proteome. Thus, both low and excessive levels of Cu may cause adverse effects in the biological systems and gastrotropin could be used as a biomarker for both situations. Gastrotropin could be considered as a detoxifying compound because of its ability to remove excessive level of Cu by inducing bile salt production (Roberts and Sarkar 2008). However, a high level of bile may induce programmed cell death in hepatocytes through the Fas ligand-independent mechanism (Sodeman *et al.* 2000; Booth *et al.* 2011).

VAT-1L plays a role in controlling the vesicular transport, storage and release of neurotransmitters from the presynaptic bulb into the synaptic cleft, and at the same time is involved in flux generation of calcium ion (Linial *et al.* 1989; Koch *et al.* 2003; Ahmed *et al.* 2013). The expression of this protein was increased along with the increase of $CuSO_4$ concentration. There were no reports regarding the interaction of VAT-1L with toxic metal ions, but other types of toxicants were reported to cause upregulation of this protein in the HEPG2 cell line and zebrafish brain proteome which were affected by hypochlorous acid and methyl parathion, respectively (San 2007; Huang and Huang 2011). Upregulation of VAT-1L was observed during wound healing (Hsiao *et al.* 2012); thus, future study is needed to clarify whether or not this protein is involved in the homeostasis mechanism through the increase of neuronal activity for biological injury recovery.

The main function of haemoglobin is as an oxygen transporter and at the same time it is also involved in the induction of erythrocyte aging, heat stabilizer, interaction with xenobiotics and catabolism sources (Giardina *et al.* 1995; Berg *et al.* 2002). Complete or adult haemoglobin is the combination of two haemoglobin subunits of α and β haemoglobin. The present study showed that the haemoglobin subunits α and β were significantly affected by $CuSO_4$, which were increasing in expression except for spot ID no. 8 where the intensity was decreased as the $CuSO_4$ concentration increased. Eyckmans *et al.* (2012) reported

the downregulation of α subunit and upregulation of β subunit in gibel carp gill proteome after being exposed to copper(ii)nitrate. The toxic effect of Cu and titanium oxide on tilapia and mouse liver proteome can be determined respectively based on the upregulation of the β subunit (Chen and Chan 2011; Jeon *et al.* 2011). Mouse injected with 50 to 70 μg/kg of Microcyctin-LR caused the upregulation of both subunits in liver proteome (Chen *et al.* 2005). The spot ID no. 8 expression profile was similar to that of Park and Lee (2014) who observed the decreasing expression of the β subunit in mouse liver proteome after being treated with dichloromethane. Handy (2003), reported that the increasing level of haemoglobin affected by Cu was related to the osmotic imbalance, and the level kept increasing until the Cu concentration reached the LC_{50} (McKim *et al.* 1970). Conversely, the decreasing haemoglobin was related to a failure of the haemoglobin gene expression caused by mitochondrial function damage from the overproduction of ROS (Niekerk *et al.* 1994; Chui *et al.* 2003; Richter *et al.* 2009). The present study suggests that α and β subunits could be considered as sensitive biomarkers of Cu or other types of toxicants as suggested by previous studies (Romero-Ruiz *et al.* 2006; Kumari *et al.* 2010; Kim *et al.* 2010).

Parvalbumin is a calcium (Ca^{2+}) binding protein involved in muscle cell relaxation after contraction (Schwaller *et al.* 1999; Murphy *et al.* 2012). $CuSO_4$ affected parvalbumin in *P. javanicus* liver proteome as indicated by the significant increase in expression in the treated groups. Sublethal concentration of Cu caused the upregulation of parvalbumin in Hepa-T1 cell and zebra fish liver cell lines (Chen and Chan 2009; Vieira *et al.* 2009). A similar protein expression was observed in liver proteome of largemouth bass after being exposed to coplanar PCB 126 (Sanchez *et al.* 2009). However, at 1.0 and 5.0 mg/L of $CuSO_4$ concentrations, the intensities of parvalbumin were drastically decreased. This situation could be explained by the overload of calcium influx in the cell, resulting in the inhibition of mitochondria respiration along with ROS production (Manzl *et al.* 2004; Jaiswal 2014). The increasing number of cell death was conclusively associated with the decrease of regulation or synthesis of parvalbumin. The present study also suggests that parvalbumin of *P. javanicus* liver proteome could be a candidate biomarker for Cu toxicity and perhaps other types of toxicants.

The expression profile was evaluated again based on the normalized values for the tested $CuSO_4$ concentration treatments. Comparisons were made between 10 identified spots from the first analysis using a dendrogram plot by means of Euclidean distance (d_e) clustering scattered data in a high dimensional Euclidean space (Fig. 4; Black). Cluster (II) and (III) were the upregulated and downregulated proteins, respectively. Parvalbumin was separated to the Cluster (I) as the unique expression of this protein was significantly different compared to (II) and (III) where the

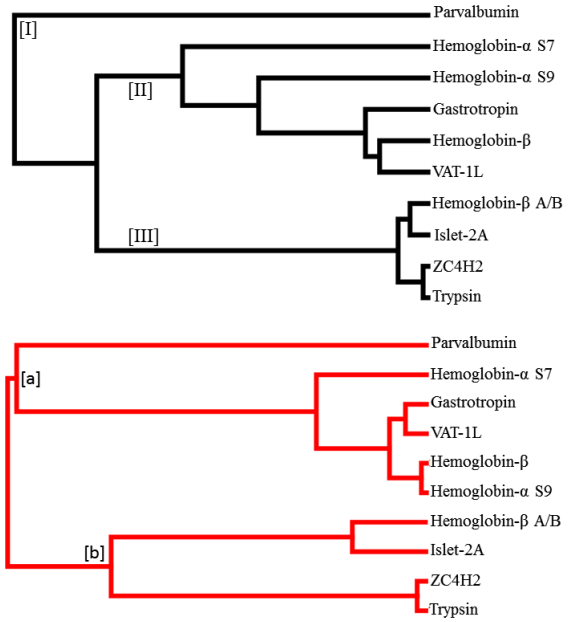

Fig. 4: Dendrogram plot on 10 PES affected by $CuSO_4$ concentration by means of Euclidean (Black) and Cosine (Red) distance. [a] and [II] marked as upregulation of protein while [b] and [III] are downregulated. [I] was no similarities in protein expression neither [II] nor [III]

normalized volume was increased at 0.5 mg/L $CuSO_4$ treatment then decreased beyond this concentration. Trypsin was clustered close to ZC4H2 protein with d_e of 0.314 suggesting an expression profile similar to proteins affected by $CuSO_4$ concentrations. The Euclidean distance between Islet2A and haemoglobin-β A/B was calculated to be $d_e = 4.897$.

Moreover, the expression profiles were analyzed using Cosine distance (d_c), in which the angle between unit vectors was counted rather than differences in magnitudes. Parvalbumin was placed in the same cluster marked by (a), which is the upregulated protein (Fig. 4; Red). The d_c also provided information about expression pattern that could be unique depending on the expression mode of action. Studies by Sabullah *et al.* (2015) showed a sensitivity comparison of acetylcholinesterase from *Periophthalmodon schlosseri* with other sources of biosensors affected by heavy metal toxicity based on hierarchical cluster analysis by means of Euclidean and Cosine distance. For example, in his paper, according to Euclidean distance, haemoglobin-β and haemoglobin-α spot no. nine (S9) were assigned far apart from each other (d_e = 2.805) originating from differences in protein expression. On the other hand, both proteins were found to be comparably close based on Cosine distance ($d_c = 0.007$) suggesting a high relationship for complete formation of adult haemoglobin as mentioned by Sankaran and Orkin (2013). The Euclidean distance showed VAT-1L to be placed in the same cluster with haemoglobin-β (d_e = 1.315), while Cosine distance showed VAT-1L to be closer to gastrotropin ($d_c = 0.012$).

Conclusion

The present study was intended as a preliminary screening on the effects of $CuSO_4$ on *P. javanicus* liver proteome and revealed 10 identified spots that were significantly affected by different $CuSO_4$ treatment concentrations. To our knowledge, no published literature has mentioned the unique expression of ZC4H2, Islet-2A and VAT-1L as shown in this study. This study suggests that these proteins from the liver and tissues of *P. javanicus* have the potential to be used as alternative biomarkers for $CuSO_4$ toxicity. Further studies using other types of toxicants on these proteins could also be fruitful.

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