

BIOREMEDIATION SCIENCE AND TECHNOLOGY RESEARCH



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A One Dimensional PAGE Studies on *Puntius javanicus* Liver Proteome Affected by Copper Toxicity

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HISTORY

Received: 11th September 2015 Received in revised form: 7th November 2015 Accepted: 15th of December 2015

KEYWORDS

Puntius javanicus 1-D PAGE native PAGE SDS-PAGE copper

ABSTRACT

Observations on the effects of copper on the liver proteome of *Puntius javanicus* based on the one dimensional PAGE was carried out. The liver was dissected from each fish, which was separately treated with different concentrations of copper sulfate ranging from 0.1 to 5.0 mg/L. The livers were extracted and one dimensional PAGE was performed under nonreducing (native) and reducing (SDS)-PAGE. Several bands were resolved in the native PAGE with probable candidates for the effect of copper observed showing an increased in the expression and downregulation strongly associated with increasing copper concentrations. This study showed that high concentrations of copper significantly alters *P. javanicus* liver at the proteome level, and preliminary screening based on one dimensional PAGE is considered rapid and simple to assess the toxicity effect of copper before more advanced and extensive assessment with a second dimensional PAGE is carried out.

INTRODUCTION

Copper (Cu) is a naturally occuring element and is widely distributed in the environment and is also found in biological systems. Cu plays a role as a co-regulator in maintaining homeotasis mechanisms, neurological activity, and cell biosynthesis [1]. However, copper exceeding the normal concentration may cause adverse effects to organisms. This problem can occur when an organism is directly and continuously exposed to Cu contamination from mining activities, industrial wastes or/and agricultural activities. Cubased products such as fertilizers and pesticides are capable of contaminating the environment through direct discharge or runoffs of excess applications [2]. The habitat of aquatic life would be affected with abnormilities rangin from the molecular, cellular to the physiological levels, and causes growth retardation, abnormal swimming performance, decreasing avoidance behaviour and loss of appetite which leads to death of aquatic organism [3-6]. Copper toxicity affects aquacultural

activity through decreasing the quantity and quality of agricultural production.

Biomarker based on the proteomic approach has been implemented to assess the stress level at the level of protein synthesis caused by environmental factors especially pollutants [7,8]. Using methods such as one (1D-PAGE) or two dimensional PAGE (2D-PAGE) analysis, the mode of action of several affected proteins in adapting, recovering or being inhibited by the effects of toxicants can be assessed [9]. Of the two methods, (1D-PAGE) can provide a rapid and simpler initial assessment of the effect of toxicants at the protein level before a complicated and time consuming 2D-PAGE is employed. In the present study, preliminary screening based on one dimensional PAGE (1D-PAGE is carried out to assess the toxic effects of Cu on the P. javanicus liver proteome using a 1D-PAGE. P. javanicus was selected as this fish is an alternative aquacultural product in Malaysia. The fish can not only be ultilised as a food source, but can be potentially use as a biomarker in monitoring programmes to evaluate environmental contamination due to toxicants.

MATERIALS AND METHODS

Specimens

P. javanicus was obtained from the Inland Fisheries Training Centre, Bukit Tinggi, Pahang and acclimatized for 15 days (12d: 12n) in aquaria containing fully areated tap water (chlorine free). The water was changed twice per week. During the experiment, each fish was separated into six groups (nine fishes per group), then copper(II)sulfate was added to the final concentrations of 0.1, 0.3 0.5, 1.0 and 5.0 mg/L [10]. Untreated group worked as a control of the study. At the end of 96 hours, the fishes were killed and the livers were dissected.

Nonreducing PAGE (Native-PAGE)

Fish liver was grounded with an extraction buffer (0.1 M sodium phosphate buffer, pH 7.5) at the ratio of 1:4 (w/v) using mortar and pestle. Secondary extraction was carried out using an UltraTurax homogenizer. The extractant was centrifuged at 10,000 xg for 15 min in 4°C. The supernatant was collected then the protein was determined using the method of Bradford [11]. PAGE was carried out based on Laemmli [12] with slight modifications. A 7% resolving gel was prepared after mixing the followings; 1.5 M Tris-HCl (pH 8.8), 30% Acrylamide/Bis (29.2/0.8), deionized water, 10% of ammonium persulfate (APS) and TEMED, while 4% stacking gel was prepared by mixing 22.3% 0.5 M Tris-HCl buffer, 15.7% of polyacrylamide solution, 61.55% of deionized water, 0.9% of APS and 0.09% TEMED. Each treated and untreated samples were separately mixed with the sample buffer (0.06 M Tris-HCl pH 6.8, 10% glycerol, 0.025% bromophenol blue) at the ratio of 3:2 (v/v). High molecular weight protein marker (thyroglobulin; 669 kDa, ferritin; 440 kDa, catalase; 232 kDa, lactate dehydrogenase; 140 kDa and albumin; 67 kDa) and samples were then loaded into the wells and electrophoresed with tank buffer under a constant current at 200 V approximately for 45 minutes or until the tracking dye had migrated out. At the end of the process, the gel was washed twice in 10 minutes with deionised buffer.

Reducing PAGE (SDS-PAGE)

One gram of liver from each treatment was grounded under N2liquid flow using mortar and pestle. The powder was mixed with the extraction buffer at the same ratio. The extraction was mixed with a sample buffer containing 0.02% SDS and 5% 2mercaptoethanol with the ratio of 3:2 (v/v) then subsequently boiled in the water bath for 10 minutes. A 10% of stacking gel was prepared containing 25, 33.3, 40.7, 0.5 and 0.05% of 1.5 M Tris-HCl (pH 8.8), 30% acrylamide/bis (29.2/0.8), deionized water, 10% of APS and TEMED respectively, with an addition of 0.1% SDS. 4% stacking was let to polymerize at the top of the stacking gel. Each samples and broad range protein markers (myosin; 200 kDa, β-galactosidase; 116.25 kDa, phosphorylase b (97.4 kDa, serum albumin; 66.2 kDa, ovalbumin; 45 kDa, carbonic anhydrase; 31 kDa, trypsin inhibitor; 21 kDa, lysozyme: 14.4 kDa, and aprotinin: 6.5 kDa) were loaded into the wells separately, followed by electrophoresis with a tank buffer containing 0.1% SDS under a constant current of 200 V for 45 min. The gel was washed twice for 30 minutes to eliminate the remaining SDS [12].

Protein visualisation

The Silver staining method was performed on both types of gels (reducing and nonreducing PAGE) based on the method of Yan et al. [13]. After an overnight incubation in a fixing solution (40% of methanol, 10% of acetic acid in deionised water), the

gel was sensitized with the solution containing 4% of sodium thiosulfate (5% stock solution), 30 % of methanol, and 6.8 % (w/v) of sodium acetate in deionised water with gentle shaking for 30 minutes. The gel was washed for five minutes with deionised water for three times. The silver reaction was performed by immersing the gel for 20 minutes in the silver nitrate solution (10 % of silver nitrate (2.5% stock solution) in deionised water). After washing twice with deionised water for a minute, the staining was developed by incubating it in a developing solution containing 2.5 % (w/v) of sodium carbonate and 0.04% of formaldehyde in deionised water. The protein bands appeared and the staining process was stopped by removing the developing solution and replacing it with a stop solution containing 1.46 % of EDTA (w/v) for 10 minutes. The gel was washed twice with deionised water for five minutes, then stored in 1% acetic acid solution for long term use.

The gel images were obtained by scanning, using a calibrated densitometer G-800 (Bio-Rad) aid with a Quantity One software for controlling the image brightness and contrast. The expression of each protein bands were quantified based on the normalised volume using the same software.

RESULTS AND DISCUSSION

A 96-hour exposure effects of Cu on the P. javanicus liver proteome at various copper concentrations treatment (Fig. 1) showed three patterns of protein bands; 1) upregulation which is the increase in expression as well as the increase of the intensity of the protein band, 2) downregulation showing the decrease of the protein's band intensity associated with the increase of Cu treatment, and 3) unchanged protein band and intensity which is considered as unaffected by the toxic effects of Cu. The were 21 horizontal lanes (labeled by arrow no. 1 to 21) containing protein bands from control to the highest concentration of Cu treated resolved in the native-PAGE gel. Each arrow shows the estimate of the protein's molecular weight calculated from the logarithmic scale plotted based on the migration of protein markers or retention factors (rf) (Fig. 2). Upregulation of protein bands was shown by arrow numbers 5, 9, 10, 12, 14 and 15 with the molecular weights estimated as 251, 132, 101, 66.9, 46.85 and 39.19 kDa, respectively. Downregulation of proteins were labed at the 6th, 7th, 13th, 16th to 21st arrows with the molecular weights estimated as 207.05, 183.85, 55.99, 32.79, 29.11, 21.63, 19.2, 17.05 and 15.14 kDa, respectively. Unaffected protein bands were labed at the 1st, 2nd, 3rd, 4th, 8th, and 11th arrows.



Fig. 1. Nonreducing PAGE gel of *P. javanicus* liver treated with different concentrations of CuSO₄. PM and C represents the protein marker and control of the data, respectively. * Downregulated protein, ** Upregulated protein.



Fig. 2. High molecular weight protein marker calibration standard plotted as logarithmic data (Log₁₀[kDa]) v retention factor (*rf*). The arrows representing protein bands are marked on the standard curve (\blacklozenge).

However, native-PAGE can only generate little information due to the limited expression of protein that can be determined through a single separation based on the protein complex migration that depends on both the charge and molecular size of each expression (protein complex band) in the globular form. The protein are in complex forms, with the exact proteins that exhibit changes in their expression cannot be fully pinpointed with a single band resolved on the native-PAGE probably contained more than one protein complex with many subunits. Thus, further separation based on the subunits composition needs to be implemented [14].

Cluster analysis was performed by means of the cosine distance (d_e) using the Mathematica version 8 software based on the expression of all 21 proteins affected by copper (**Fig.** 3). The red line shows the unaffected proteins which were clustered together with protein bands no. 1 and 2 showing the closest cosine distance at d_e = 0.0002. The majority of the downregulated proteins was placed together at cluster no. I. Conversely, the downregulated protein bands no. 17 and 21 were clustered with the upregulated protein (cluster no. II) in which the expression pattern showed a sudden drop at the copper concentrations of 0.1 and 1.0 mg/L, respectively. The expression then increased drastically suggesting a strong relationship of these proteins to adapt and defend against toxic copper concentrations.

The protein expression for protein bands no. 14 and 15 were slightly increased but statistically there was no significant difference(p < 0.05) at the copper concentrations of 0.3 and 0.5 mg/L, and both were clustered together with the downregulated protein group and a cosine distance was calculated at d_c =0.0017. These results hypothesize a comparable expression of each protein affected by copper toxicity. Thus the tested protein is applicable to be used as a rapid tool in determining copper exposure.



Fig. 3. Dendrogram plot on 21 protein bands affected by copper concentrations by means of Cosine distance. The red line marks unaffected protein. Cluster [I] and [II] marks the downregulated and upregulated protein expression, respectively, affected by copper.

Several protein bands exhibited significant effects of copper concentration both under reducing or denaturing conditions and the expected broad protein monomer would be determined from lower to the higher range of protein size. There were abundant numbers of protein bands which are very hard to be marked one by one. Thus, the protein bands were grouped in a box based on three types of protein expression labelled from 1 to 8 (**Fig. 4**).

Group number three appears to have no significant changes in its protein expression. However box numbers 1, 2, 4 and 8 showed an increase in protein expression where the molecular weights were estimated ranging from 60 to 91, 45 to 60, 21 to 31, and below 6.5 kDa, respectively. Conversely, the protein bands which indicate downregulation were labelled box numbers 5, 6 and 7 in which the molecular weight ranges from 6.5 to 21 kDa.

Several unaffected proteins to any types of stressor were to be related perhaps to the structural types of protein as studied by Wang et al. [15] which reported keratin and lamin were not affected by inorganic mercury exposure. Overexpression of protein is due to fulfilling the biological demand. Most of the upregulated proteins caused by the toxic effect of copper is related to detoxification, antioxidant and induction of delerious effects, which include proteins such as gluthathione Stransferase, metallothionine, superoxide dismutase and catalase [16,17].

Elmore, [16] mentioned the development of proteins called vascular endothelial growth factors and cyclin in damage restoration, toxicant neutralisation and removal via digestion or autophagic activity. Downregulation of protein associated with the inhibition or malfunction such as studied by Hartwig [18] and Aiba et al., [19] explained the decrease expression of zinc finger proteins were due to the dislocation of zinc and replacement by cadmium or cobalt which consequently results in an inactive and loss of the binding activity on DNA.



Fig. 4: SDS Page gel resolved with *P. javanicus* liver proteome from each different treatment of Cu concentration. The red box shows a group of unaffected proteins by CuSO₄ concentration. * Downregulated protein, ** upregulated protein.

Compared to native PAGE, SDS-PAGE displayed a lower degree of uncertainty which is more advantageous in predicting a single protein especially on a unique protein subunit expression. Unfortunately, an individual band can still contain more than one type of overlapping protein. Thus, another separation step needs to be carried out to clearly distinguish the interfering protein with the same molecular mass. 2D-PAGE is further better separation of the protein abundance via pI in the second dimension. Despite this, the one dimensional SDS PAGE is a simpler and faster method as a preliminary way to assess protein changes due to exposure to toxicants.

CONCLUSION

In this study, using either native or SDS-PAGE as a preliminary screening method to prove the toxic effects of copper as an alternative method to 2D-PAGE plays an important role as rapid assessment method and as an indicator to ensure the successful performance of the 2D-PAGE. The relationship of native or SDS-PAGE with 2D-PAGE is due to the number of bands resolved which is supposed to correspond to the existing number of protein spots resolved on 2D gel and is normally more than the amount of bands expressed on 1D-PAGE gel. One of the reasons to perform a preliminary screening by native-PAGE and SDS-PAGE before proceeding with another step of separation via accurate 2D-PAGE analysis is to avoid time consumption and reagent wastages due to a failed 2D-PAGE, which is common.

ACKNOWLEDGEMENT

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