On the 15th day of study, the animals were euthanized by cervical dislocation and post-mortem examinations were carried out soon after death. The visceral organs, namely liver, kidney, spleen, stomach, small intestine, large intestine, caecum, pancreas, urinary bladder, lungs, heart, brain, skeletal muscles and reproductive organs were carefully dissected out and fixed in 10% formal saline for histopathological examinations. Micro sections of 5 μm cut from the tissues embedded in paraffin wax were stained with haematoxylin and eosin (H&E) and examined under light microscope for histopathological changes[11].

As indicated in Table 1, the experimental animals did not show severe clinical signs suggestive of toxicity during the experiment. However, one animal (treatment III, male-2) fed with the highest dose of test substance developed diarrhoea during the latter part of the experiment. Apart from mild to moderate dilatation of the intestine due to gas accumulation (tympanic changes) in four animals, the gross post-mortem findings did not show any significant damage. These observations indicate that oral administration of water extract of *A. sessilis* does not cause acute clinical disease in Swiss mice. However, histopathological changes were found in liver and kidney specimens of the mice fed with the plant extract (Table 1). In contrast, mice in the control group were found with no histopathological changes or damage in their body (Figure 1 *a* and *b*). Moreover, the histological lesions were relatively severe in treatment-III (highest dose) when compared with the other groups. These lesions were characterized by moderate to severe hepatocyte degeneration in centrilobular area associated with widespread sinusoidal congestion and focal hepatocellular necrosis (Figure 2 *a* and *b*). Four of the five mice in treatment-II had mild to moderate hepatocyte degeneration and necrosis. The degenerative changes of hepatocytes found in treatment-I was not consistent and did not appear in all the animals. As shown in Table 1, kidney lesions were found only in animals of treatments II and III. Kidney specimens of treatment III showed moderate degeneration of renal tubular cells and necrosis (Figure 3), whereas treatment II showed only mild degenerative changes of the renal tubular cells.

These findings indicate that oral administration of water extract of *A. sessilis* in high doses leads to histopathological changes in the liver and kidney tissues of Swiss mice. An earlier study carried out using an alkaloidal extract of *A. sessilis* administered intra-peritoneally resulted in alteration of liver and kidney functions in Swiss mice[9]. Based on all these findings, it can be concluded that changes in the liver and kidney functions are due to lesions in the liver and kidney caused by cytotoxic substance/s in *A. sessilis*. However, further investigations are necessary in order to understand the long-term effect of the consumption of cooked *A. sessilis* in smaller quantities.

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Preliminary analysis of cuprome of *Anabaena doliolum* using two-dimensional gel electrophoresis

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This study provides first-hand information on the initial characterization of copper-induced changes in the global proteome (hereafter called cuprome) of *Anabaena doliolum* subjected to short- and long-term treatments. PD Quest analysis revealed that out of 215 protein spots in the control, 79 showed alterations (26 up- and 36 down-regulation, and 5 up- and 12 down-regulation res-

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pectively, after 24 and 168 h of Cu treatment) in their expression pattern. The short-term (24 h) and long-term (168 h) treatments induced respectively, 158 and 96 proteins. Of the 158 newly induced proteins, 30 were found to sustain the long-term treatment. In view of the appearance of two sets of proteins, there appears a need to carry out short- and long-term proteome analysis for getting a holistic view of the proteome of cyanobacteria subjected to abiotic stress.

Keywords: *Anabaena doliolum*, copper, cuprome, two-dimensional gel electrophoresis.

ANABAENA DOLIOLUM, a photoautotrophic cyanobacterium, constitutes an important fraction of the N_2-fixing microflora of the paddies. Increased production of rice for meeting the food demand of the ever-growing population requires enormous use of fertilizers and metal-containing pesticides, which result in heavy metal contamination of paddy fields and the cyanobacteria inhabiting therein. Among different heavy metals, Cu assumes special significance due to its involvement in metalloenzymes, including cytochrome c oxidase, lysyl oxidase, SOD and plastocyanins[1] at low concentration. At high concentration, however, Cu exerts toxicity[2] not only to PSII but catalyses the synthesis of reactive oxygen species which damage proteins and lipids[3,4].

Cyanobacteria are known to adapt to environmental stresses by suitably modifying their proteome[5] and 2DE (two-dimensional gel electrophoresis) has emerged as a powerful technique for resolving the proteome of different organisms[6]. Bhagwat and Apte[7] successfully analysed the stress stimulon of salinity-tolerant *Anabaena* sp. L31 subjected to heat, salinity and osmotic stress and demonstrated induction of two different sets of polypeptides. One set with four polypeptides was common for all the three stresses studied, while the other set of four polypeptides was unique to heat stress only. Cd-stress stimulon in *Escherichia coli*[8] and UV-B stress stimulon in desiccation tolerant *Nostoc commune*[9] have also been successfully investigated using 2DE. The later two studies demonstrated induction of two sets of stress proteins, e.g. (i) after short-term, and (ii) after long-term exposure of the organism. Further, Sazuka[10] analysed the proteome of *Anabaena* sp. PCC 7120 using 2DE and characterized 123 most abundant proteins.

Suroz and Palinska[11] have studied the effect of different doses of copper on the SDS–PAGE protein profile of *Anabaena flos-aquae* and demonstrated down-regulation of the synthesis of a large number of proteins and up-regulation of only one protein of 55 kDa. In view of the above, there appears a complete lack of information on the copper-induced stress stimulon of microbes in general and N_2-fixing cyanobacteria in particular. Thus for the proper understanding of the stress stimulon of any organism, there is a need to identify (i) the early and the late shock proteins, (ii) involvement of early shock proteins in late acclimation process, i.e. how many of the early shock proteins are retained during the long-term treatment, (iii) how far the two kinds of proteins are related to each other, and (iv) induction of novel proteins, if any, by the stress in question. It is believed that the information generated by (i) and (ii) is essential for complete analysis of proteome. The present study was, therefore, undertaken to employ 2DE to characterize (i) that subset of proteins which depict up- or down-regulation against copper stress, (ii) the effect of short- and long-term copper treatment on induction of proteins, (iii) the number of proteins of the transient phase which resist long term copper toxicity, and (iv) preliminary characterization of proteins involved in adaptation of cyanobacteria to copper stress.

The test cyanobacterium, *A. doliolum* Bharadwaja was grown axenically in a modified Allen and Arnon[12] medium buffered with Tris/HCl at 24 ± 2°C under daylight fluorescent tubes emitting 72 µmol photon m^{-2} s^{-1} PAR (photosynthetically active radiation) light intensity with a photoperiod of 14:10 h at pH 7.5. The cultures were shaken by hand 2–3 times daily.

The 20 µM Cu selected for the experiment was LC_{75} concentration, as determined by the plate colony count method of Rai and Raizada[13]. This concentration produced maximum change in the protein profile in SDS–PAGE (data not shown). Time durations selected for this work were 24 h (did not show much alteration in the absorbance) and 168 h (7 days) showing maximum toxicity which was also confirmed by SDS–PAGE protein profile (data not shown).

Protein extraction was performed using a modified protocol given in Wagner *et al.*[14] *A. doliolum* cells harvested by centrifugation (starting culture volume 500 ml and absorbance 0.5 at 663 nm) were washed with Tris-HCl buffer (pH 8.0) and re-suspended in 5 ml extraction buffer containing 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$ and 10 mM KCl. Cells ground in liquid nitrogen were centrifuged at 9000 rcf for 1 h. The supernatant so obtained was subjected to overnight precipitation using 10% TCA in acetone and centrifuged at 5800 rcf for 15 min to recover the protein. Additional washing with acetone was performed to remove TCA. The pellet was air-dried and suspended in sample-loading buffer containing 8 M urea, 2% CHAPS, 1% DTT (DL-Dithiothreitol) and 0.8% ampholyte (pH 5–7). This suspension was centrifuged and passed through Sephadex G-25 column to remove undissolved material and salt before using for 2DE analysis.

Two-dimensional gel electrophoresis was performed according to the method of O'Farrell[15] with minor modification using PROTEAN II xi Cell (Bio-Rad). Isoelectric focusing was done in 15 × 1.5 mm vertical glass tubes with 4.5% T gels containing 5% carrier ampholyte (four parts pH 3–10 and one part pH 5–7). This was given a prerun for 190 Vh before loading with samples containing 50 µg protein. Isoelectric focusing was conducted overnight for a total of 14,000 Vh. The tubes were extruded and equilibrated first in sample-loading buffer containing 2%

SDS, 50 mM Tris HCl (pH 8.8), 6 M urea, 30% glycerol, 0.002% bromophenol blue and 1% DTT followed by 2.5% 2-iodoacetamide (instead of 1% DTT) for 15 min each. This gel was then transferred onto the second dimension having 12% T resolving and 4% T stacking gels and run at 20 mA for 12 h. Protein spots stained with coomassie brilliant blue R-250 were characterized using a marker protein (SIGMA) coelectrophoresed alongside the second-dimension gel.

The 2DE images were scanned using gel documentation system (Bio-Rad). Image analysis, including alignment and matching was done with the PD Quest software (Bio-Rad). All experiments were performed in three independent replicates and only those spots present in at least two gels of the independent set were taken for analysis.

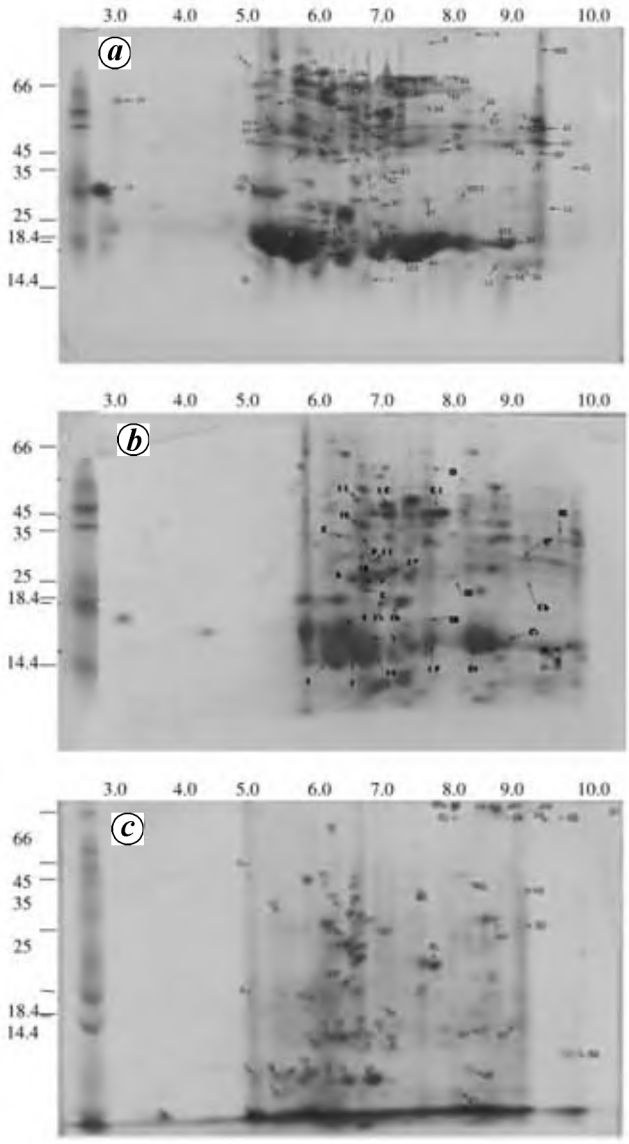

Figure 1. Two-dimensional gel images of total cell protein from *Anabaena doliolum* exposed to (***a***) control, (***b***) 20 μM Cu for 24 h, and (***c***) 20 μM Cu for 168 h.

Figure 1 shows the 2DE patterns of the total cell protein of *A. doliolum* exposed to 20 μM copper for 24 (Figure 1 *b*) and 168 h (Figure 1 *c*). Digital imaging revealed around 215, 220 and 162 spots in the control, 24 h and 168 h (7 days) copper treatments respectively (Figure 1 *a–c*). The number of spots observed in the control gel finds support from the report of Sazuka[10], who not only observed 123 most abundant proteins of *Anabaena* PCC7120, but also established their gene linkage map. Further, the quantitative changes observed were accompanied by changes in the intensity of the protein spots as well (Figure 2). Thus the cuprome of an organism may be defined as that subset of proteins which respond to copper stress by either being up- or down-regulated. Figure 2 depicts a three-dimensional scatter plot of the cuprome in which the *X*-axis shows the pI, *Y*-axis molecular weight, and *Z*-axis intensity of the spots. Almost 90% of the cuprome was found clustered in the range of 10–40 kDa and 5–9 pI.

Figure 3 demonstrates clubbing of the cuprome into distinct subsets. The area represented by $A \cap B$ contains a set of 62 proteins (26 up- and 36 down-regulated) induced by short-term Cu treatment. This set conceivably corresponds to the class of general stress proteins (Gsps)[16] which are likely to provide a non-specific protection. The nature and mode of action of these Gsps are still poorly understood. These proteins are difficult to study because of their pleiotropic functions with respect to carbon fixation, stress tolerance, nutrient starvation, etc.[16]. Further, six of the 26 spots stated above (spot nos IV, V, VI, X, XV and XVI in Figure 1 *a*) persisted even after 168 h (7 days) of Cu treatment. On the basis of molecular weight and pI three of these, i.e. IV, V and XV showed resem-

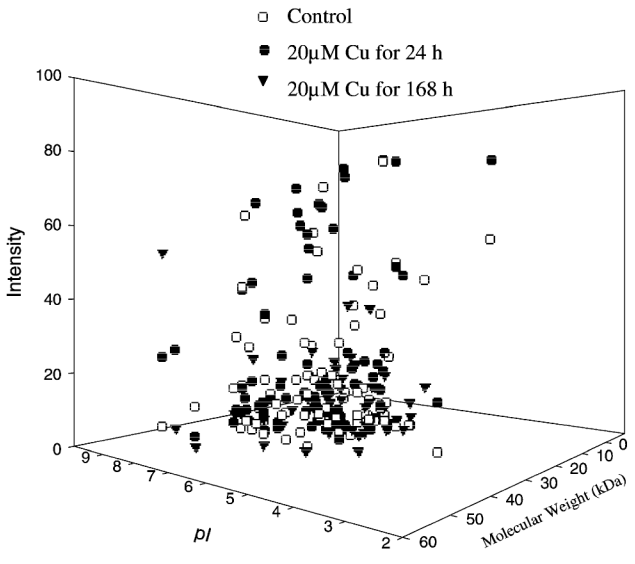

Figure 2. Three-dimensional scatter diagram of selected protein spots from two-dimensional electrophoretic gels with *X*, *Y* and *Z*-axes depicting pI, molecular weight and intensity of protein spots.

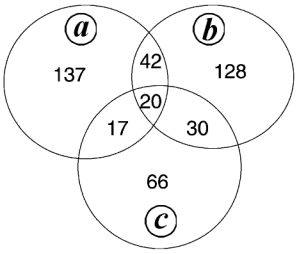

Figure 3. Venn diagram representation of different subsets of proteins expressed in the different treatments. ***a***, Control; ***b***, 20 µM Cu for 24 h; ***c***, 20 µM Cu for 168 h.

blance with 33, 24 and 17 kDa proteins. Since Cu is known to be toxic to PSII, synthesis of these polypeptides supposedly protects the O_2-evolving complex[17] of the cyanobacterium.

A second subset as represented by *B–A* (B minus A) contains 158 newly induced proteins following 24 h Cu treatment. Of these, 128 gave a fleeting appearance and only 30 could endure 168 h (7 days) of copper toxicity. The transiently appearing proteins may be involved in the rapid adaptation of cells to the changed environment. These observations find support from the work of Apte and Bhagwat[5], who reported time-dependent differences in the protein pattern following exposure to different levels of salinity.

Further, a third set of 17 proteins represented by $(A \cap C) - (A \cap B \cap C)$ are those synthesized only after 168 h Cu treatment. Of these, five showed more than two-fold induction (spot nos 6, 9, 10, 11 and 12 in Figure 1 *a*). These may be specific to offering tolerance against copper stress. Finally a fourth subset of 66 proteins as represented by $C - (A \cap B)$ are those synthesized *de novo* after 7 days of copper treatment. Of these, spot 61 (pI ≈ 8.0 and mol. wt 78 kDa) gave a remarkably prominent appearance. These, however, did not depict close resemblance with the already known proteins of the *Anabaena* sp. Thus the present data unfold two interesting sets of information. The first is a two-step expression of copper toxicity. This involves development of transient proteins, conceivably responsible for the maintenance of the homeostasis as well as adaptation of the cyanobacterium against Cu stress. This is followed by the emergence of different sets of proteins resisting the long-term copper treatment. The second set of information is a 30% resemblance in the protein profiles of short- and long-term Cu treatments. This indicates that the test cyanobacterium first employs a general strategy, e.g. production of general stress proteins for adaptation against stress followed by the induction of stress-specific proteins.

This study suggests that for a holistic view of proteins induced by abiotic stresses, short- and long-term treatments should be taken into account. It also provides a preliminary distinction between transient and late acclimation proteins of *A. doliolum*. The present study provides interesting insights for embarking on the proteomics work under abiotic stress. Efforts are on to perform the MALDI/TOF analysis of selected proteins, which may give a more clear-cut view of the cuprome of *A. doliolum*.

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