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Proteomics study revealed altered proteome of *Dichogaster curgensis* upon exposure to fly ash

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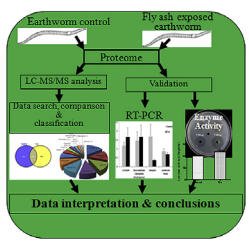
HIGHLIGHTS

- Proteomics of fly ash exposed *Dichogaster curgensis* were reported.
- Label free quantitation was used to determine protein abundance.
- Proteomics data revealed oxidative and cellular stress in FA exposed earthworms.
- Proteomic analysis revealed induced protein synthesis and mis-folding of proteins.
- Comet assay suggested DNA-protein cross link in FA exposed earthworms.

GRAPHICAL ABSTRACT



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ABSTRACT

Fly ash is toxic and its escalating use as a soil amendment and disposal by dumping into environment is receiving alarming attention due to its impact on environment. Proteomics technology is being used for environmental studies since proteins respond rapidly when an organism is exposed to a toxicant, and hence soil engineers such as earthworms are used as model organisms to assess the toxic effects of soil toxicants. This study adopted proteomics technology and profiled proteome of earthworm *Dichogaster curgensis* that was exposed to fly ash, with main aim to elucidate fly ash effects on cellular and metabolic pathways. The functional classification of identified proteins revealed carbohydrate metabolism (14.36%), genetic information processing (15.02%), folding, sorting and degradation (10.83%), replication and repair (3.95%); environmental information processing (2.19%), signal transduction (9.61%), transport and catabolism (17.27%), energy metabolism (6.69%), etc. in the proteome. Proteomics data and functional assays revealed that the exposure of earthworm to fly ash induced protein synthesis, up-regulation of gluconeogenesis, disturbed energy metabolism, oxidative and cellular stress, and mis-folding of proteins. The regulation of ubiquitination, proteasome and modified alkaline comet assay in earthworm coelomocytes suggested DNA-protein cross link affecting chromatin remodeling and protein folding.

1. Introduction

Soil is the mixture of minerals, organic matter, liquids, and myriad organisms that together support life and plays major role in water filtering; hence, it has to be maintained to ensure a

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sustainable environment (Sheaffer and Moncada, 2012). However, soil pollution with heavy metals, polycyclic aromatic hydrocarbon, pesticides and herbicides, toxic industrial waste, etc. have become a widespread environmental problem throughout the world. In India, since it's one of the largest energy consumers (ranking 6th in the world) and largest reservoir of coal (4th in the world), the electricity generation is mainly dependent upon the coal-based thermal power plants (Pandey et al., 2011). The combustion of coal in coal-thermal power plants results in generation of fly ash (FA), which is hazardous to health as it contains potentially toxic heavy metals (Sharma et al., 1989; Ayuke et al., 2011a; Markad et al., 2012), and radioactive elements (Mandal and Sengupta, 2003; Papastefanou, 2008). Electricity consumption is expected to continue to increase due to increasing industrialization, urbanization, and a growing population. The current alternative technologies cannot easily substitute existing coal-thermal power plants and hence waste generation i.e. FA will keep accumulating and dumping.

More than 50% of the fly ash generated is utilized in agricultural sector or construction purposes, while remaining portion is disposed in ash ponds near the power plants occupying more than 65,000 acres of land in India alone (Pandey and Singh, 2010; Pandey et al., 2011). The repetitive application of FA as soil amendment and due to dumping of large quantities of FA, the quantity of heavy metals and other toxicants in soil increases significantly which results into health and environmental hazards. In comparison to organic compounds, heavy metals do not get decompose and hence they accumulate in plants and soil organisms like earthworms (Malandrino et al., 2011). These heavy metals have been shown to cause mortality, reduce fertility and are known to be detrimental to earthworms (Calisi et al., 2013). Toxicological effects of fly ash and their leachates is well documented in various organisms like fish (Ali et al., 2004), earthworms (Manerikar et al., 2008; Markad et al., 2012; Maity et al., 2009; Grumiaux et al., 2010) and plants (Chakraborty and Mukherjee, 2009). Thus, FA globally started gaining an alarming attention as an anthropogenic source of various toxic chemicals and it kindled risk assessment and soil biomonitoring.

Soil has been characterized using physical and chemical parameters to access toxic pollutants (Morvan et al., 2008), but soil microorganisms, termites and earthworms that strongly impact on the soil environment have also been used as a soil quality indicators (Ayuke et al., 2011a, 2011b; Zida et al., 2011). Earthworms are a key representatives of soil fauna and are essential in maintaining soil fertility through their burrowing, ingestion, and excretion (Edwards, 2004). They are sensitive to soil pollutants, extreme soil conditions including temperatures, soil moisture, soil texture, organic matter content, pH and poor drainage (Breure et al., 2005; Römbke et al., 2005; Forey et al., 2011; Zida et al., 2011; Fournier et al., 2012). Considering their high density in fertile soils, quick response to natural and anthropic stress and soil toxicants; researchers are keen to exploit earthworms as an indicator for soil pollution (Paoletti, 1999; Lavelle and Spain, 2001; Sousa et al., 2006; Tondoh et al., 2007).

Researchers are focusing on the identification of specific earthworm biomarkers related to specific soil toxicants e.g. metallothionein for heavy metals like cadmium monitoring (Xu et al., 2005; Amara et al., 2008). Enzyme activity and transcription of phase II detoxification superfamily glutathione S-transferases (GST) in earthworm has been presumed as a biomarker of soil toxin including industrial metals and pesticides (Aly and Schröder, 2008; Maity et al., 2008). Metallothionein response of *Lampito mauritii* was assessed and found that they were influenced by multiple environmental factors and are not specific; thus singular biomarker studies have some drawbacks (Maity et al., 2009). In order to gain a more detailed toxicological comprehension, there is an increasing demand for application of proteomic analysis in ecotoxicology i.e. an ecotoxicoproteomics approach (Wang et al., 2010b). Toxicological response of earthworm after phenanthrene and cadmium exposure were analyzed by using two dimensional electrophoresis (2DE) (Kuperman et al., 2003; Wang et al., 2010b; Wu et al., 2013). The combination of sub-proteomics, bioinformatics, and biochemical assay has been used to characterize glutathione S-transferase of earthworm *Lumbricus rubellus* (LaCourse et al., 2009). Earthworm acts as a major ecosystem engineers, ingests and excrete soil, and thus soil microbial community remain as an integral part of the earthworms. Protein expression changes measured in tissue or body fluid or at the level of whole organism exposed to soil pollutants could provide evidence of toxic effects (Lagadic et al., 2000). Limited literature exists on the effects of FA on protein expressions in earthworms. In India, *Dichogaster curgensis* has been used for conversion of solid wastes into compost. Therefore, this study adopted label free quantitative proteomics technology to explore protein expression profile in earthworm, *D. curgensis* exposed to FA. This study could establish specific protein abundance profile or protein biomarkers that could act as an early warning signal of FA contamination of soil.

2. Materials and methods

2.1. Earthworms and exposure conditions

Earthworm *Dichogaster curgensis* was a generous gift from University of Agricultural Sciences, Gandhi Krishi Vignan Kendra, Bengaluru, India. Earthworm *D. curgensis* was maintained in laboratory on hand collected, dried, homogenized cattle manure with 12/12 h dark–light cycle, 40–50% humidity, and temperature at 22 ± 2 °C. The adult healthy worms with average weight of about 250–300 mg with well-developed clitellum were selected for the study. Earthworms were exposed to fly ash (40%) for 14 days in polythene culture pots (20 cm × 10 cm × 8 cm) under laboratory conditions of temperature (22 ± 2 °C), moisture content (40%), and photoperiod (12 h light: 12 h dark). Physico-chemical characteristics of the experimental sets were reported in our earlier study (Markad et al., 2012). The concentration and duration of exposure were selected based on our previous work (Markad et al., 2012).

2.2. Protein extraction and protein digestion

Proteins from earthworms were extracted using trichloroacetic acid/acetone (TCA–A) procedure as described previously (Wang et al., 2010a). Briefly, earthworm tissue (1 g) was ground to a fine powder in liquid nitrogen with mortar and pestle. The powder was re-suspended in 10 ml ice-cold protein extraction buffer (20% TCA-A/0.1% DTT) and proteins were precipitated overnight at −20 °C. Protein pellets obtained after centrifugation at 12,000 × g at 4 °C were washed with ice-cold methanol followed by multiple washes of ice-cold acetone. The pellets were vacuum-dried and re-solubilized in lysis buffer (7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT). Protein content was determined by the Bradford's method.

Proteins (40 μg) from each test sample were separated on 12% SDS-PAGE (12% polyacrylamide separating gel with 4% stacking gel) at 120 V, and bands were visualized with Commassie Brilliant Blue R250 staining. In-gel digestion was performed following the protocol reported earlier (Ravindran et al., 2012; Adav et al., 2013). In brief, each sample lane was separately sliced into eight portions (fractions), further cut into pieces (approximately 1 mm^2), washed with 75% acetonitrile (ACN) containing 25 mM ammonium bicarbonate buffer (Sigma, St. Louis, MO, USA), and de-stained. The de-