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Expression of cytosolic and thiolated proteome of *Musca domestica* larvae under oxidative challenge

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Abstract. The study was aimed to investigate the expression of cytosolic and thiolated proteins of *Musca domestica* larvae under oxidative stress. Proteins from acute treatment of hydrogen peroxide (LC_{50} = 21.52% (v/v)) on 3rd stage larvae of housefly were extracted and purified using an activated Thiol Sepharose® for thiolated protein purification. Two dimensional gel electrophoresis was used for visualizing and analyzing expression of cytosolic and thiolated proteins. Protein spots with more than 5 fold of expression change were identified using liquid chromatography- tandem mass spectrometry (LC-MS/MS). The cytosolic proteins were actin, tropomyosin, ubiquitin, arginine kinase, pheromone binding protein/general odorant binding protein, and ATP: guanidino phosphotransferase. The thiolated proteins with more than 5 fold change in expression as an effect to the acute treatment were fructose bisphosphate aldolase, short chain dehydrogenase and lactate/malate dehydrogenase. The proteins identified in the study should provide vital information for future reference in oxidative stress defence and response occurring in houseflies.

INTRODUCTION

Musca domestica (1758, Linnaeus) (Diptera: Muscidae), commonly known as housefly, is a synanthropic insect that lives in the urban areas where there are high densities of human waste as their food source (Dahlem, 2003). An adult housefly completes its metamorphosis from egg to adult stage. Housefly breeds extremely fast when compared to other species of flies. Female houseflies lay 500 eggs, in 5 to 6 batches in her lifespan, with 75 – 100 eggs deposited each batch in the span of just 3 to 4 days.

Houseflies are known to be vectors of various diseases of over 30 bacteria including *Heliobacter pylori*, *Escherichia coli*, and even methicilin resistant *Staphylococcus* and tiarcilin resistant *Pseudomonas* (Boulesteix *et al*., 2005; Grubel *et al*., 1997; Szalanski *et al*., 2004). Houseflies carry viruses such as Newcastle disease virus (Barin *et al*., 2010). Resistance of houseflies to insecticides happen across the globe (Marçon *et al*., 2003; Scott *et al*., 2000). For housefly larvae, Kristensen & Jespersen (2003) found resistance strains against insect growth regulators. Molecular biologists are highly interested on houseflies due to their growing resistance to insecticides. Scott *et al*. (2009) suggested *Musca domestica* as a model organism for resistance studies and development of new insecticides. Insecticides including pyrethroids (Kale *et al*., 1999), organophosphates (Lukaszewicz-Hussain, 2010), and organochlorines (Koner *et al*., 1998) have known to be inducing oxidative stress.

Hydrogen peroxide are one of the non-radical reactive oxygen species. Despite its oxidizing capacities, hydrogen peroxide is constantly produced in cellular functions. In *Musca domestica*, GST gene family (Yin *et al*., 2000), SOD gene (Dabas *et al*., 2012) and glutathione peroxidase (Simmons *et al*., 1987) involve in housefly defence against

hydrogen peroxide. Cysteine residues, the only sulfhydryl/thiol (SH) – bearing amino acid, have high reactivity and unique redox properties, forming key catalytic components of enzyme on the active site (Barford, 2004). Oxidative thiol modification by hydrogen peroxide are found to occur in enzymes and proteins such as glyceraldehyde-3-phosphate dehydrogenase (GapDH) (Brandes *et al*., 2007), PTP-1B (protein tyrosine phosphatases-1B) (van Montfort *et al*., 2003) and p53 tumor suppressing factor (Velu *et al*., 2007).

Despite the immunity and insecticide tolerance of *Musca domestica*, which are vital as a model for insect biochemistry and physiology, the genome project for housefly were only initiated at 2009 (Scott *et al*., 2009). More importantly, to the best of our knowledge, only a handful of *Musca domestica* related proteomics research are available. However, during the past 5 years there is an increasing interest to understand the inner molecular workings of this insect, notably the completion of sequencing of the full genome of *Musca domestica* by Scott *et al*. (2014). Combining thiol protein trapping techniques (Hu *et al*., 2010), 2D-gel electrophoresis (Klose, 1975; O'Farrell, 1975) and the LC-MS/MS identification, we investigated the cytosolic proteins and thiolated proteins alike. The aim of this study was to evaluate and identify the possible responsive proteins when larvae are under oxidative stress.

MATERIALS AND METHODS

Chemicals

Unless otherwise stipulated, highest grade of chemicals was used in this research. Chemicals related with 2D gel electrophoresis were of analytical grade purchased from BioRad Laboratories, (Hercules, CA, USA) and GE Healthcare (Little Chalfont, United Kingdom). Activated Thiol-Sepharose R 4B was purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

Musca domestica adult and larvae cultivation

Adult *Musca domestica* samples were obtained from Vector Control Research Unit, World Health Organization, University of Science Malaysia, Penang, Malaysia. The flies were reared in modified plastic cages at room temperature. The adults fed on sugar and milk powder mixed on 1:1 ratio. Housefly larval was laid on surface made of Ramy Feeds hamster pellet (Bengy) soaked with distilled water in a 1:1.5 ratio. For the experiments, 3rd stage larvae were tested.

Oxidative challenge of 3rd stage larvae and determination of toxicity parameters

6 g of mouse pellet was mixed with 10ml of solution of different concentrations of H_2O_2 solution (concentration ranging from 15%, 17.5%, 20%, 22.5% and 25% (v/v). 300 individuals of 3 day-old larvae (3rd stage) (Kočišová *et al*., 2004) were collected, and placed to each medium of different H_2O_2 concentrations and left to feed for 24 hours. Larvae which were observed not moving were considered dead by the acute treatment. The number mortalities of the larvae were counted and subjected to PROBIT analysis (Finney, 1947). The concentration of the hydrogen peroxide which caused 50% mortalities (LC_{50}) of larvae population was determined and used for proteomic investigation.

Determination of lipid peroxidation

The larvae were homogenized in homogenizing buffer (0.1 M NaH_2PO_4 buffer, pH 7.5, 1.3 mM EDTA, 0.1 mM EDTA, 1% protease inhibitor, and traces of phenylthiourea) using HG-15D homgenizer (WiseTis R) (Tedesco *et al*., 2010). Samples were centrifuged at 3000 x *g* for 20 minutes and then derivatized in 1 ml of reaction mixture containing 10.3 mM 1- methyl-2-phenylindole which was dissolved in acetonitrile: methanol (3 : 1 v/v) with 32% (v/v) HCl. The malondialdehyde (MDA) standard curve was produced to determine the internal concentration of MDA

in nmol/g (wet weight). The absorbance of unknowns and standard curve were read at 586 nm.

Preparation of protein lysate and thiol protein purification

ReadyPrep™ Protein Extraction Kit (Cytoplasmic/Nuclear) (Bio-Rad) was used to extract cytosolic proteins from the homogenate, according to the manufacturer's protocol. 50 µl of protein lysate were aliquoted and kept at -80°C until further analysis.

Purification of thiols protein was performed as described by Hu *et al.* (2010). Protein lysate (with suitable protein content) was incubated with 5 M urea for 10 minutes at room temperature. 20 mg of activated Thiol-Sepharose® 4B (Sigma-Aldrich) and 200 µl binding buffer solution (0.1 M Tris-HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA) were added. The samples were incubated on ice for 1.5 hours and shaken for every 15 minutes to ensure maximum binding. The gel matrix containing bound proteins were washed 8 times with 500 µl of binding buffer and the unbound proteins were discarded. Finally, 200 µl of binding buffer containing 25mM DTT was added and the matrix was incubated on ice for 1 hour with frequent gentle shaking to release all bound thiol-containing proteins. The mixture was centrifuged at 11000 x *g* for 3 minutes and the protein thiol-containing proteins were collected and kept at -80°C for further analysis.

Protein quantitation

Protein quantitation of the sample lysate for both total proteome and thiol protein purified samples was done by using 2-D Quant Kit (GE Healthcare) accordingly to the manufacturer's protocol. A bovine serum albumin solution of 2 mg/ml was diluted into protein content ranging from 0 µg to 50 µg. 500 µl of precipitant was added to each tube, vortexed and incubated for 2-3 minutes at room temperature. 500 µl of co-precipitant was later added and vortexed. The tubes were centrifuged for 10,000 × *g* for 5 minutes to sediment the protein. The precipitant and co-precipitant were decanted and removed. 100 µl of copper solution and 400 µl of deionized water were added and vortexed to dissolve the protein precipitate. 1 ml of the working colour reagent, prepared by mixing 100 parts of colour reagent A and 1 part of colour reagent B of the kit was added into each tube, inverted and incubated in room temperature for 15-20 minutes. Absorbance of 480 nm was read for each tube with water as reference.

Two dimensional gel electrophoresis

40 µl of protein samples (protein lysate for cytosolic or the prepared thiol-containing proteins) were dissolved in 45 µl of solubilizing buffer (8M urea, 4% CHAPS, 65mM DTT, 3M thiourea, trace bromophenol blue) and 40 µl of rehydration solution (8M urea, 2% CHAPS, 0.2 % (w/v) DTT, 2% carrier ampholytes (pH 3-10), traces of bromophenol blue). The mixtures were applied onto 7 cm Immobiline™ Drystrips, pH 3-10 NL IPG strip (GE Healthcare). The strips were rehydrated overnight for 18 hours. Isoelectric focusing were done in Multiphor III (GE Healthcare) under the voltage setup: Step 1 (Gradient): Voltage: 200V; Time: 1 minute; Current: 5mA; Power: 2W. Step 2 (Gradient): Voltage: 3500V; Time: 1.5 hours; Current: 5mA; Power: 2W. Step 3 (Gradient): Voltage: 3500V; Time: 1.5 hours; Current: 5mA; Power: 2W.

The temperature of the focusing was controlled by the thermostatic circulator Multitemp III (GE Healthcare) at 16°C, and the power supply was provided by EPS 3500 XL (GE Healthcare). The isoelectric focusing setup was covered in PlusOne Drystrip Cover Fluid (GE Healthcare). The strips were then equilibrated in 2.5ml of equilibration buffer I (1.5 M Tris- HCl buffer, 6 M urea, 4.7 M glycerol, 2% (w/v) SDS, 16.2 mM DTT) and equilibration buffer II (1.5 M Tris-HCl buffer, 6 M Urea, 4.7 M glycerol, 2% (w/v) SDS, 0.243 mM 2- iodoacetamide) for 15 minutes.

SDS-PAGE on the strips was done in 12% resolving and 4% stacking gel, embedded with 0.5% agarose containing trace bromophenol blue. The electrophoresis processes were performed as described by the instructions of the Mini-Protean II Tetra Cell (Bio-Rad) with voltage set to 150V.

Electrophoresis was performed in a descending manner with 1X SDS-PAGE running buffer (Bio-Rad) until the dye front reached at the end of the gel. BenchMark™ Unstained Protein Ladder (Thermo Fisher Scientific Inc.) was concurrently run as standards for estimating molecular weight of protein spots. After SDS-gel electrophoresis, the gel was stained and visualized with Coomassie Brilliant Blue R-250 staining solution (5% (w/v) Coomassie Brilliant Blue, 85% (v/v) ortho-phosphoric acid and ammonium sulphate) for 4 days and destained with 20% (v/v) methanol until the gel background was clear and clear spots were observed.

Differential expression analysis

The gel images were processed using the Image Scanner III (GE Healthcare) and Labscan software (GE Healthcare). All of the spot analyses were done using PDQuest software (Bio-Rad) of which the spot detection was done according to Gaussian distribution. The spot intensities were averaged and comparative evaluation on the spot location between control and treated sample were investigated. Ratio of intensity of the same spot in two different gels was determined and the change of intensity was interpreted as change in protein expression. T-test analysis of at 90% significance ($p < 0.10$) was also performed using the software. Spots with more than 5 fold of change difference were excised for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. To ensure reliability of the spot identity, spots of similar location on both control and treated gels were identified.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis

Destaining of the gel plugs were done using 15 mM potassium ferricyanide in 50 mM sodium thiosulphate pentahydrate until transparent. Using 10 mM DTT in 100 mM ammonium bicarbonate and 55 mM iodoacetamide in 100 mM ammonium bicarbonate, the plugs were further reduced and alkylated. Then, the plugs were washed with 50% (v/v) acetonitrile in 100 mM ammonium bicarbonate and 100% (v/v) acetonitrile and followed with dehydration using vacuum centrifugation. The dried plugs were incubated overnight in 25 µl of 6 ng/µl trypsin (Sigma-Aldrich) in 50 mM ammonium bicarbonate at 37°C. The peptides were dried in SpeedVac™ (Thermo Scientific) and desalted with ZipTipR C18 resin (Merck Milipore). The peptides were dried again and reconstituted in 4 µl 0.1% (v/v) formic acid in water. The LC-MS/MS system used was the Agilent 1200 HPLC-Chip/MS Interface, coupled with Agilent 6520 Accurate-Mass Q-TOF LC/MS+ ESI. The column used was C18, 160 nl enrichment column and 75 µm x 150 mm analytical column (Agilent). Flow rate employed was 4 µl/min from Agilent 1200 Series Capillary pump and 0.3 µl/min from Agilent 1200 Series Nano Pump. Sample injection volume was 2 µl. Solvent A was 90% acetonitrile in water and solution B was 0.1% formic acid. Flow rate employed was 4 µl/min from Agilent 1200 Series Capillary pump and 0.3 µl/min from Agilent 1200 Series Nano Pump. The solvent gradient employed a stop time at 47 minutes and variation of solvent gradient was optimized for peptide analysis. The spectra were acquired via two modes with the MS scan range of 110-3000 m/z and in the MS/MS the scan range 50-3000 m/z was set. For the precursor selection I, the absorbance threshold was 200. Precursor selection II was performed to select fragments of charge states of 2, 3, or more than 3. Data was processed with Agilent Spectrum Mill MS Proteomics Workbench software packages. The scan range for MH+ ion was set from 600 to 4000 Da. Database search was done on SwissProt and National Center for Biotechnology Information (NCBI). %SPI for peptides were toggled to be more than 60% and the average distinct summed MS/MS search score over number of peptides was more than 11. The database selected aimed at current *Musca domestica* species at Uniprot and SwissprotKB database. The selection of protein entries was based by the notion that the identified entries' MW to be closest to the approximated MW in the 2D gels.

RESULTS

The probit analysis has indicated that H_2O_2 solution at concentration of 21.5% (v/v) would cause 50% of mortalities of 3rd stage *Musca domestica* larvae. During the acute treatment of larvae with H_2O_2 (at LC_{50}), our investigation has also indicated that lipid peroxidation product MDA increased 10 times in the treated larvae. This suggested that the oxidative stress has occurred and changes in the protein expression were due to the effect of the acute peroxide treatment. Proteomic analysis revealed that in the event of acute hydrogen peroxide treatment, 3 proteins were significantly down-regulated more than 5 times ($p<0.10$) (actin, ATP: guanido phosphotransferase, ubiquitin) and up-regulated (tropomyosin, arginine kinase, PBP/GOBP family protein). In thiolated proteome, a short chain/alcohol dehydrogenase like protein and lactate dehydrogenase were down-regulated more than 5 fold. The study also demonstrated that arginine kinase and fructose-bisphosphate aldolase were upregulated more than 5 fold. The changes of the selected proteins were visualized in Figure 1. Table 1 summarizes the quantitative analysis of the change in expression and the identification of the spots. As the % SPI for peptides were more than 60% and the average distinct summed MS/MS search score over number of peptides was more than 11 (distinct peptide score that implies a significant protein identification), our results are sufficiently valid. In our study, the protein expression was solely investigated to imply the correlation between acute treatment and expression of cytosolic and thiolated proteins.

DISCUSSIONS

Actin levels were reduced while tropomyosin levels were elevated in hydrogen peroxide challenged housefly larvae. Actin (monomer; G-actin: globular actin) polymerizes into filaments better known as F-actin (filamentous actin) under physiological conditions to form cytoskeleton in cells (Huber *et al.*, 2013). Tropomyosin on the other hand stabilizes actin filament and mediates actin binding proteins in muscular tissues (Cooper, 2002). Muscle enhancer MEF2 which regulates *Drosophila* TmI gene in muscles in larvae (Lin & Stroti, 1997) is switched on during oxidative stress. Clam *Chamaelea gallina* which were exposed to Aroclor 1254 and copper(II), had the actin gene downregulated while the putative isoforms of tropomyosin were upregulated (Rodríguez Ortega *et al.*, 2003).

Oxidative challenge on housefly larvae might also affect the cellular ubiquitin proteasomal activity. Based on the results, ubiquitin abundance reduced in the event of the oxidative stress (7.90 times down-regulation, Spot C, Figure 1), suggesting a disruption or a shift of homeostasis of ubiquitin. A 76-residue polypeptide, ubiquitin is highly conserved and present in all eukaryotes (Goldstein *et al.*, 1975). The decrease of monomeric ubiquitin could be accounted to the attack on the Cys residues in ubiquitin conjugating enzymes in ubiquitin-proteasome pathway, regulated via E1 (ubiquitin-activating enzymes), E2 (ubiquitin conjugating enzymes) and E3 (ubiquitin ligase enzymes). E1 activates Gly residue of ubiquitin to Cys residue to generate high energy thiolester intermediate (E1~S ubiquitin), while E2 relays the activated ubiquitin from E1 via an internal Cys residue as well (Ye & Rape, 2009). Decrease of ubiquitin pool in oxidatively challenged housefly larvae might be also due to the viability of deubiquitinating enzymes (DUBs), which catalyze the process of breaking down ubiquitinated substrates/free polyubiquitin chain in the cell (Amerik & Hochstrasser, 2004) and the synthesis of ubiquitin (Redman & Rechsteiner, 1989). DUB's catalytic site consists of highly conserved regions of histidine and cysteine boxes (Reyes-Turcu *et al.*, 2006). It is conceivable that oxidative damage occurred in Cys residues on the ubiquitin conjugating and deubiquitination enzymes, causing the decrease in ubiquitin.

Fast alternative energy metabolism and ATP buffering possibly occurred during oxidative stress. Arginine kinase (8.97 fold increase, spot E, Figure 1) catalyzes

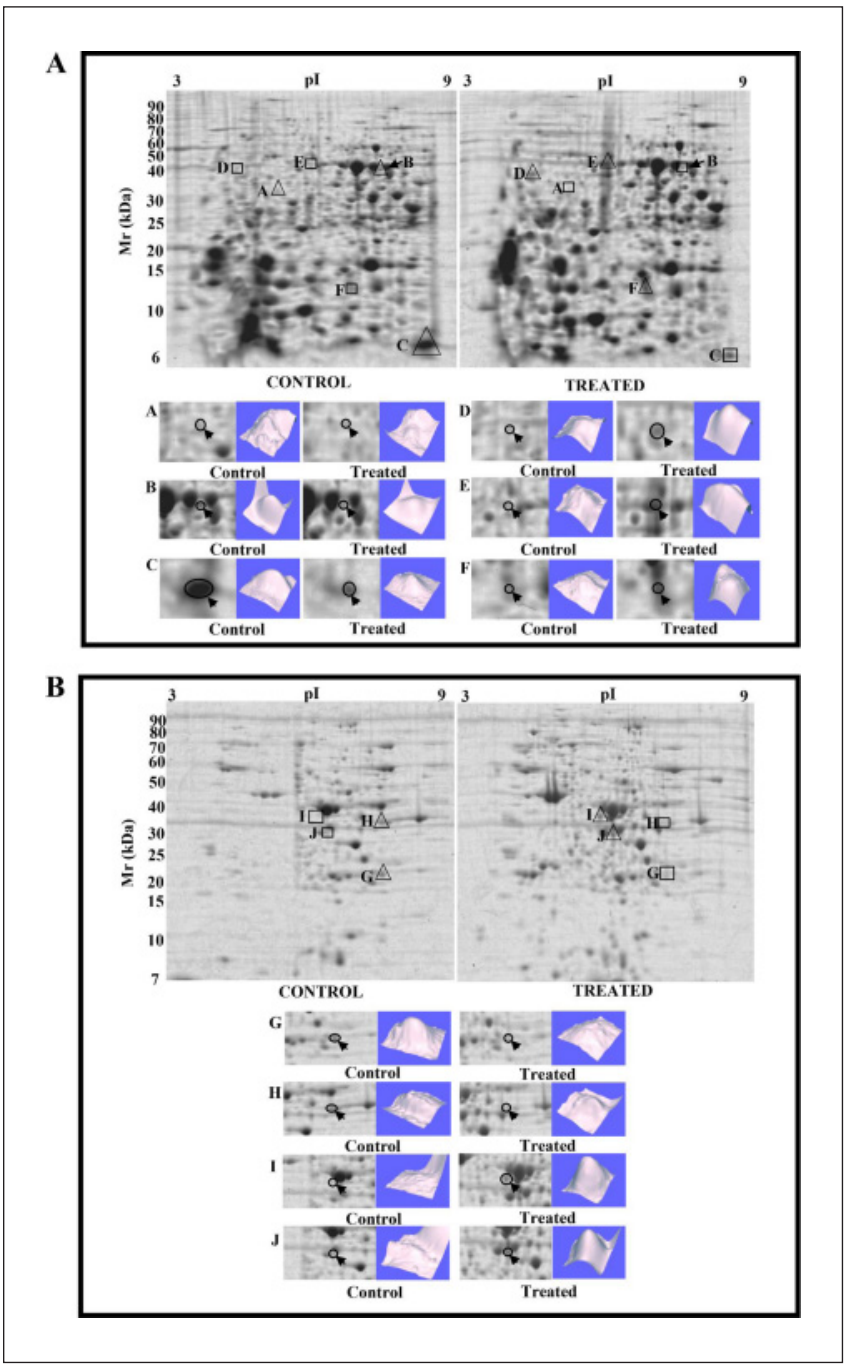

Figure 1. Spots position of proteome expression profile on more than 5x fold change in of *Musca domestica* 3rd stage larvae.

Box A shows comparative investigation of cytosolic proteome profile of which spots were designated from A-F in Box A. The 3-D presentation of the compared spots was made to visually compare the spot intensity. Box B indicates thiolated protein spots designated from G-J that had expression change more than 5 fold. The spots were either identified in Δ and □ on both gels to indicate changes (> 5 fold change) in protein expression of the same protein in control and treated gel. The respective 3D images of the comparative protein spots were shown below the gel images. Arrows indicate the location of the spots on two different gels. Gels were stained with Coomassie Blue.

Table 1. Shows the change in expression fold and the identification of protein spots assigned in Figure 1. Arrows indicate increase (↑) or decrease (↓) of expression

Spot	Fold change	Database accession number	Protein Name	Mr/pI (Predicted)	Number of distinct peptides	% aa coverage	Search Score
A	7.29 ↓	399943076	actin	34900.0/5.2	4	11.7	49.78
B	7.74 ↓	3999399272	ATP: guanido phosphotransferase	43100.0/NA	10	27.5	148.21
C	7.90 ↓	Q45TR8	Ubiquitin (Fragment)	6100.0/NA	6	64.4	99.06
D	39.08 ↑	39939626	Tropomyosin	40200.0/4.7	8	25.2	90.43
E	8.97 ↑	430769005	Arginine kinase	44600/5.6	27	62.6	419.39
F	5.82 ↑	399940676	PBP/GOBP family protein	11500.0/5.9	10	47.1	148.21
G	5.67 ↓	557782744	alcohol dehydrogenase-like isoform X2	21500/6.82	2	13.7	25.1
H	8.05 ↓	399938050	Lactate/malate dehydrogenase	34900/6.7	5	16.2	80.4
I	8.77 ↑	430769005	Arginine kinase	37200/5.8	16	40.4	235.9
J	6.14 ↑	399943052	Fructose-bisphosphate aldolase class-1	31800.0/6.0	4	18.1	58.39

*Note: Distinct Summed MS/MS Search Score with more than 11 were of significant protein identification according the Agilent Spectrum Mill MS Proteomics.

the reversible reaction of transferring phosphorus group between MgATP and arginine acting as energy-storing phosphagen. Phosphoarginine is one of the phosphagen in living systems, smaller than ATP and able to diffuse to provide fast energy supply (Ellington, 2001) enabling cells to restore ATP levels during bursts of cellular events such as in nerves and muscles (Hird, 1986). Miranda *et al.* (2006) demonstrated the increment of arginine kinase rates increased during hypoxia. Arginine kinase was detected in high fold changes in cytosolic proteome (8.97 fold) and thiolated proteome (8.77 fold). This suggested that *Musca domestica* larvae highly expresses arginine kinase, at the same time highly protects this protein from oxidative modification by hydrogen peroxide. Thus, we suggested that arginine kinase could be vital in *Musca domestica* larvae's energy metabolism response. utilizing phosphoarginine to provide alternative cellular energy source and ATP buffering during oxidative stress.

Other protein responses in high level expression during oxidative challenge including a protein from ATP:guanidino phosphotranferase family, which expressed 7.74 fold higher than in control replicates (Spot B, Figure 1). ATP:guanidino phosphotransferases catalyzes the transfer of phosphate between phosphagens and ATP (Stein *et al.*, 1990). Arginine kinase exists in mitochondrial and cytoplasm in isoforms (Munneke & Collier, 1988). Thus, it is important to identify and characterize specific isoform that participated in responses to oxidative stress. PBP/GOBP protein family (pheromone binding protein/ general odorant binding protein) from Spot F (Figure 1) has shown an increase of 5.82 fold of expression in treated samples. The protein is localized in the aqueous fluid around olfactory sensory dendrites to bind and transport hydrophobic odorants (Vogt & Riddiford, 1981). PBP, binds specifically towards pheromone (Du & Prestwich, 1995) while GOBP associate with general-odorant sensitive neurons (Vogt *et al.*, 1991). Further investigation is needed to understand its correlation to oxidative stress.

Arginine kinase are present in the thiol proteome profile (Spot I, Figure 1), possibly due to its conserved residue Cys271 at the active site of arginine kinase (Zhou *et al.*, 1998). The overexpression of arginine kinase as an effect of oxidative stress has been discussed. As the non-thiolated protein was removed, we observed enhancement of a glycolytic enzyme during acute peroxide treatment. There was a 6.14 fold increase (Spot J, Figure 1) in expression of fructose bisphosphate aldolase in treated samples. The enzyme catalyzes the forward reaction of aldol splitting of fructose 1,6-bisphosphate to dihydroxyacetone phosphate to glyceraldehyde 3-phosphate and the reverse reaction (Gardberg *et al.*, 2011). Sequence analysis by Brenner-Holzach (1979) however showed no exposed thiol groups of *Drosophila melanogaster*'s fructose bisphosphate aldolase. Interestingly, Bourrett *et al.* (2001) reported that fructose bisphosphate aldolase of *Borrelia burgdorferi* was able to sustain nitrosative damage on its cysteine thiols. Changes involved in glycolytic enzymes expression is an interesting entry point to further understand oxidative stress responses in energy metabolism.

Spot G (Figure 1) is a short-chain alcohol dehydrogenase, which is a ubiquitous NAD(P)(H)- dependent enzyme. Our study indicated a decrease of the protein expression under acute treatment at 5.67 fold. It was easily purified due to the fact that it has Cys-218 (which is in proximity to NAD+ binding site and does not have any catalytic properties) (Chen *et al.*, 1990) and two other solvent facing cysteine residues (Mayoral *et al.*, 2013). Proteins that belong to this family have low pairwise sequence identity (15%–30%) (Filling *et al.*, 2002). Thus, it is still far from conclusive that which short chain dehydrogenase of 3rd stage larvae of *Musca domestica* was responsive towards oxidative stress. Present in 8.05 fold decrease in expression was a protein that belongs to lactate/malate dehydrogenase family (Spot H, Figure 1). Both lactate/malate dehydrogenases catalyze the conversion of 2-hydroxy acids to the corresponding 2-keto acids

(Birktoft *et al*., 1982). Lactate dehydrogenases are important enzymes in anaerobic metabolism, by interconverting lactate to pyruvate utilizing NAD(P)/NAD(P)(H) (Holbrook *et al*., 1975). A highly-conserved cysteine residue, Cys-165 located at the proximity of co-substrate binding site (Taylor *et al*., 1973) possibly allowed its selection into our purification protocol. Oxidative stress has been shown to overexpress lactate dehydrogenase favouring anaerobic respiration instead of bringing it down (Oliveira & Oliveira, 2002). Decrease of lactate dehydrogenase in our experiment might be just a physiological event of the extracellular leakage rather than metabolic, caused by the damage of plasma membrane during cell death (Bagchi *et al*., 1995).

CONCLUSION

We have successfully discovered potential protein biomarkers during acute hydrogen peroxide treatment performed on *Musca domestica* 3rd stage larvae via two dimensional gel electrophoresis and LC-MS/MS identification. Protein with high-fold changes in expression identified in the proteome involved in biological processes such as cytoskeletal network (actin and tropomyosin), ubiquitin proteasomal activity (ubiquitin), and alternative energy metabolism (arginine kinase). A glycolytic enzyme (fructose bisphosphate aldolase) and arginine kinase changed in expression in hydrogen peroxide treated samples from the thiol proteome profile. We identified other proteins from cytosolic proteome profile (PBP/GOBP and ATP:guanidino phosphotranferase family) and thiol proteome profile (lactate/malate dehydrogenase and PBP/GOBP family protein), however understanding their role in oxidative stress response requires further investigation. We hope through this discovery we can provide more insights on oxidative stress defence in houseflies in future.

Conflict of Interest

There is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

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