[Frontiers in Bioscience E4, 1780-1786, January 1, 2012]

Proteomic analysis of peach fruit moth larvae treated with phosphine

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1. ABSTRACT

Phosphine has been used worldwide for the control of stored-product insects for many years. However, the molecular mechanism of its toxicity is not clearly understood. In the current study, larvae of the peach fruit moth were fumigated with phosphine. Proteomic analysis was then performed to identify the regulated proteins. Our results confirmed the phosphine toxicity on the peach fruit moth. The median lethal time LT50 was 38.5 h at 330 ppm at 25 °C. During fumigation, the respiration of the peach fruit moth was extremely inhibited. Of the 26 regulated proteins, 16 were identified by MALDI-TOF mass spectrometry after a 24 h treatment. The proteins were classified as related to metabolism (25%), anti-oxidation (6%), signal transduction (38%), or defense (19%). The rest (13%) were unclassified. Phosphine regulation of ATP and glutathione contents, as well as of ATP synthase and glutathione S-transferase 2 activities were confirmed by enzyme activity analysis. These results demonstrate that complex transcriptional regulations underlie phosphine fumigation. New theories on the mechanism of phosphine toxicity may also be established based on these results.

2. INTRODUCTION

Phosphine has been employed as a fumigant for more than half a century (1). It is by far the only fumigant with worldwide registration because of its low residues, low cost, and ease of use (2). Unfortunately, the long-term use of phosphine increases the risk of resistance in pest populations. Indeed, the emergence of high-level resistances among many pest insects over the last few decades has been reported (3,4). For this reason, studies on the mechanism of phosphine toxicity are receiving significant attention.

Phosphine fumigation causes physiological changes in numerous species and tissues. Among such changes, the most widely reported are respiration inhibition and ATP depletion (5,6,7,8). Initially, phosphine was proposed as capable of inhibiting cytochrome c oxidase (Complex IV) like cyanide does (9). However, further *in vivo* studies on insects, mites, rats, and humans demonstrated that phosphine only partially inhibits Complex IV activity, and that other targets must exist (5,6,7,8). Other various physiological changes resulting

from phosphine fumigation, such as lipid peroxidation (10,11), peroxidase inhibition (2), and glutathione depletion (12,13), have been further revealed. Another recent report also shows that Ferrtin-1 expression, which contributes to the maintenance of iron homeostasis, is regulated by phosphine (14). These observations indicate that phosphine toxicity is related to complex effects and molecular mechanisms, which require further investigations.

The peach fruit moth, Carposina sasakii Matsumura, is a major insect pest in apples, pears, hawthorns, and other rosaceous fruits (15). Phosphine toxicity against the larvae of the peach fruit moth has been determined in our previous work (16). In the present study, we combined two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) analyses to detect and identify differentially expressed proteins in phosphine-treated and untreated peach moth larvae. The results reveal that phosphine regulates several important proteins involved in the metabolism, signal transduction, and defense mechanism of the pest. The identification of these proteins confirms that complex transcriptional regulations underlie phosphine fumigation, and provides new theories on the phosphine toxicity mechanism.

3. MATERIALS AND METHODS

3.1. Preparation of test insects

Golden Delicious apples, which were infected with the peach fruit moth, were collected from an orchard in Liaoning, China. The apples were placed in a plant growth chamber (KBWF720, Binder, Germany) at 25 ± 0.5 °C, 60% ± 5% relative humidity, and light-dark cycles of 14.5:9.5 h until mature larvae escaped from the apple.

3.2. Phosphine treatment

For the fumigation treatments, 1.08% diluted pure phosphine was balanced with 98.02% nitrogen in a gas cylinder from Beiyang Special Gas Inc. (Beijing, China). The gas mixture was released into a 1 L Tedlar sample bag (Delin, Dalian, China) and stored at 25 °C before fumigation. After temperature equilibration, the fumigating bottles (Z263036-1PAK, Sigma, Germany) were sealed with a valve (33304, Sigma, Germany). About 8 mL of phosphine gas was then injected into the bottles after a small amount of air was removed. The lid stopcock was removed to bring the bottle pressure back to normal, and was returned afterwards. The bottles were transported in a constant temperature incubator (KBF720, Binder, Germany) to start the fumigation. The phosphine and CO_2 concentrations were monitored by a gas chromatograph (Agilent 6890N, Propark Q column; oven = 70 °C, thermal conductivity detector = 250 °C) as previously described (16).

For the toxicity test, groups of 50 mature peach fruit moth larvae were exposed to 330 ppm of phosphine for 12, 24, 48, 72, 96, and 120 h at 25 °C. The treated and control vials were aerated for 1 h after fumigation. The larvae were then transferred to plastic boxes with moist sawdust, and were kept under rearing conditions for 14 d to calculate mortality rates. The experiment was thrice replicated independently.

3.3. Sample preparation

After being fumigated with 330 ppm of phosphine for 24 h, the treated and untreated insects were collected and were immediately plunged into liquid nitrogen. The samples were grinded and then suspended in 10% trichloroacetic acid. After 16 h of precipitation, the collected proteins were pelletized by centrifugation at 12,000 × g for 10 min. Washing with 3 volumes of ice cold acetone for 2 h at –20 °C followed. The dried protein extracts were subsequently treated with lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 40 mM dithiothreitol, and 2% v/v pharmalyte; pH 4–7; GE Healthcare) at room temperature for 4 h with stirring. Centrifugation at 100,000 × g at 4 °C for 1 h followed. Finally, the protein concentrations were determined using a 2-DE Quant Kit (GE Healthcare), and the samples were stored at -80 °C until further use.

3.4. 2-DE and protein identification

Isoelectric focusing was performed on precast 24 cm immobilized gradient strips (pH 4–7). Approximately 250 µg of protein samples were loaded onto each strips. The 2-DE analysis, gel staining, and protein identification were performed as previously described (17).

3.5. Enzyme activity assays

ATP was extracted as described by Nicholas *et al.* (18) and was measured using an ATP Bioluminescence Assay Kit (Roche). Glutathione content was determined as described by Ji and Fu (19). ATP synthase activity was analyzed using kits from the Jiancheng Company (Nanjing, China) based on continuous spectrophotometric assays. Glutathione S-transferase (GST) activity was determined by monitoring its absorbance at 340 nm, as described by Kampranis *et al.* (20). All experiments were thrice replicated independently. Untreated insects were used as controls.

4. RESULTS

4.1. Phosphine toxicity on the peach fruit moth

Peach fruit moth larvae were exposed to 330 ppm of phosphine for different durations. Mortality rates increased with increased exposure time. Almost all the test insects died after 120 h of fumigation, and the median lethal time LT50 was 38.5 h (Figure 1). The mortality rate was only 12% after 24 h of fumigation, and the larvae in this group were used for further analysis. The phosphine and CO_2 concentrations for the first 24 h were also determined. With increased exposure time, the concentration of phosphine decreased, whereas that of CO_2 increased. The respiration rate of the treated insects was about 55% lower than that of the untreated insects, indicating the respiratory inhibition effect of phosphine (Figure 2).

4.2. Differential proteomic profiles of the peach fruit moth in response to phosphine treatment

A total of 26 proteins in the peach fruit moth larvae were selected for their differential regulations under phosphine influence and 16 of them were successfully identified. The differential expression profile of these

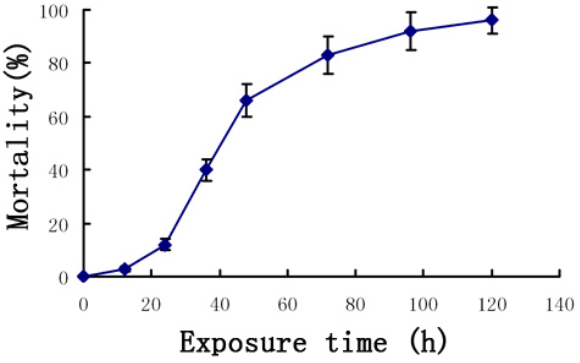

Figure 1. Mortality rates of phosphine-treated peach fruit moth larvae. The phosphine concentration used was 330 ppm. All values are presented as the average of three replicates.

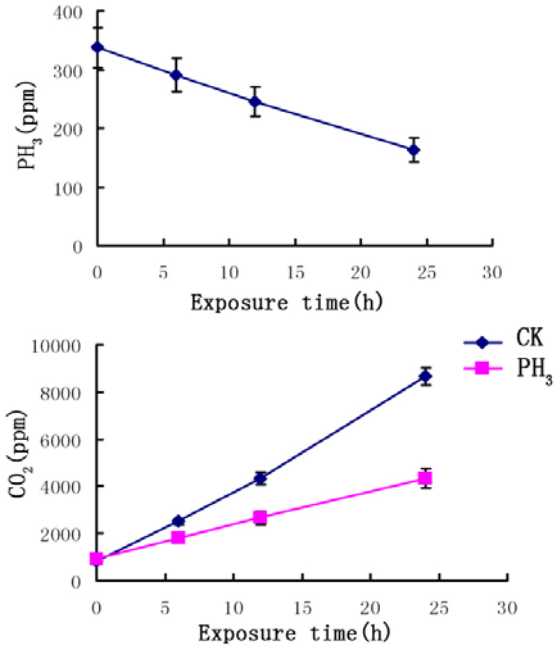

Figure 2. Phosphine and CO_2 concentrations during fumigation. All values are presented as the average of three replicates.

proteins potentially relates to the phosphine toxicity mechanism. The profiles showed increased levels of 8 proteins and decreased levels of the other 8. Table 1 lists these proteins as identified by matrix-assisted laser desorption/ionisation-time of flight MS. The proteins were grouped into 5 according to their predicted molecular functions: "metabolism" (4 proteins), "anti-oxidation" (1), "signal transduction" (6), "defense" (3), and "unclassified" (2). Figure 3 shows the 2-DE analysis results for these proteins, whose spots are marked correspondingly.

4.3. Enzyme activity analysis

The nucleotide sequences of the genes that encode the regulated proteins in the peach fruit moth are still unknown. Hence, the ATP synthase and GST activities, as well as the ATP and glutathione contents of the treated and untreated pests were determined to confirm the proteomic results. ATP synthase and GST activities changed similarly with protein level changes, and ATP and glutathione contents decreased (Figure 4).

5. DISCUSSION

In the present study, 2-DE and MS analyses were combined to provide the differential global expression profiles of the proteins in the peach fruit moth following phosphine treatment. Phosphine toxicity was confirmed, and 16 differently functional proteins related to this toxicity were identified.

All the 4 proteins in the metabolism group were down-regulated. Protein 1 was identified as ATP synthase, which drives ATP synthesis (21). Decreased ATP synthase activity and content were also detected. ATP is a key factor in energy metabolism, and ATP depletion has been deemed an important mechanism of phosphine poisoning (5,6,7,8). Based on these results, we believe that ATP synthase down-regulation causing decreased ATP synthesis is one possible mechanism of phosphine toxicity. Proteins 2, 5, and 7 were identified, respectively, as an oxidoreductase that catalyzes electron transfer from one molecule to another, a glutamate decarboxylase that participates in amino acid metabolism (22), and a pyrimidine-nucleoside phosphorylase that participates in pyrimidine metabolism. These enzymes are involved in many other basic metabolic procedures as well. Therefore, their down-regulation could be the molecular basis of the physiological inhibition effects of phosphine.

Oxidative damage is one of the most important mechanisms of phosphine toxicity, and glutathione plays an important role in anti-oxidation (12). GST catalyzes the conjugation of reduced glutathione with electrophilic centers on a wide variety of substrates. GST also detoxifies endogenous compounds such as peroxidised lipids (23). Our results showed that GST expression was down-regulated by phosphine. This result was confirmed by decreased GST activity and glutathione content. Considering that phosphine inhibits the antioxidants catalase and peroxidase in many insects (2), we believe that phosphine inhibited the glutathione-mediated anti-oxidation system in the present study by repressing GST expression.

Six proteins with signal transduction functions were regulated by phosphine. Let-363, a member of the LEThal family, is involved in regulating development and aging in Caenorhabditis elegans (24,25). Protein-tyrosine kinase, a protein kinase subclass, functions as an "on–off" switch in many cellular events, such as cell development and cell cycle control (26). This kinase also acts as a receptor of extracellular signals transmitted through the cell membrane to the cytoplasm (27). Pleckstrin-2, supposedly a transcription factor, selectively interacts with phosphatidylinositol 3-kinase lipid products as well as regulates actin organization and cell spreading (28). Histidine kinases, a class of typically transmembrane proteins, play important roles in signal transduction across

Table 1. Proteomic analysis results of differently expressed phosphine-regulated proteins in peach fruit moth larvae

Spot No.	Gene Name	Genbank No.	Protein Name	pI/MW (kDa)	Coverage (%)	Peptide Matched	Exp. value	Mascot Score	Change folder[a]
Classification: Metabolism									
1	AtpA	gi|2665936	ATP synthase subunit alpha	6.1/56.5	30	14	0.0024	96	0.15
2	PRK	gi|158319123	putative oxidoreductase	6.5/46.6	27	7	0.11	70	0.17
5	GadB	gi|31758	glutamate decarboxylase	6.6/6.8	28	9	0.21	61	0.35
7	PNP	gi|168185409	pyrimidine-nucleoside phosphorylase	5.2/47.4	20	9	0.47	74	0.38
Anti-oxidation									
8	GST2	gi|112361467	glutathione S-transferase 2	6.1/27.6	23	6	0.14	69	0.20
Signal transduction									
3	TEL1	gi|32563905	LEThal family member (let-363)	5.0/94.5	17	29	0.032	85	6.85
4	PTK2B	gi|27886588	protein-tyrosine kinase 2-beta isoform b	5.7/112	25	22	0.011	73	3.10
6	PLEK2	gi|39644830	Pleckstrin-2	9.5/35.5	21	7	0.01	74	0.40
9	HisKA	gi|256785282	histidine kinase	5.4/53.9	19	9	0.15	69	0.37
11	EGF_CA	gi|21410823	EGF-like repeats and discoidin I-like domains 3	7.1/55.1	25	12	0.027	69	2.69
12	PKc_like	gi|18375646	tyrosine-protein phosphatase non-receptor type 13 isoform 1	6.0/278	21	7	0.14	64	2.98
Defense									
14	PspA	gi|218549010	phage shock protein	5.5/25.5	56	13	0.0029	96	12.5
15	Msh2	gi|212528164	DNA mismatch repair protein Msh2, putative	5.7/107	25	14	0.011	60	15.1
21	P0	gi|37359627	ribosomal P0 protein	5.7/34.2	25	9	0.0021	97	7.58
Unclassified									
17	-	gi|119590320	hCG2041594	7.4/9.8	31	5	0.029	69	2.97
19	-	gi|158256198	unnamed protein	7.4/25.7	42	10	0.017	73	0.31

[a] Average folder between phosphine-treated and untreated insects calculated for at least three replicate gels (a change folder >1 means up-regulation after phosphine treatment, and <1 means down-regulation; Student's t-test, $p < 0.05$)

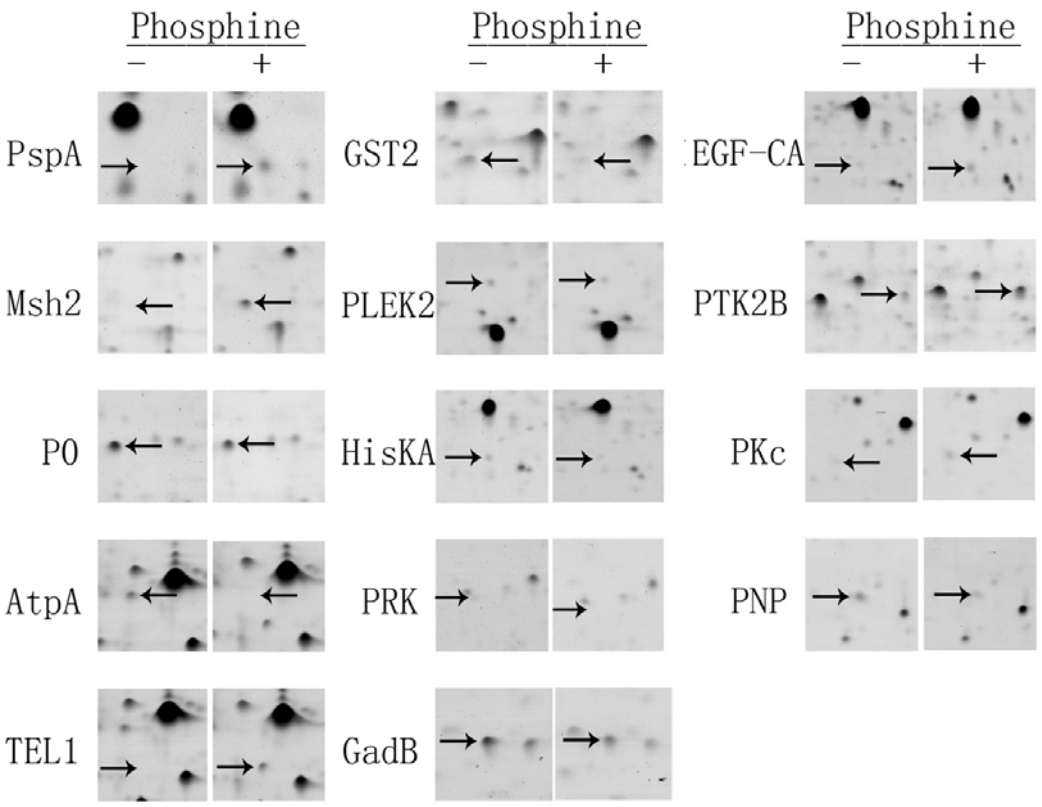

Figure 3. Spots of the differently expressed proteins identified in two-dimensional electrophoresis gels. Phosphine (–) refers to untreated insects, and phosphine (+) refers to insects treated with 330 ppm of phosphine for 24 h.

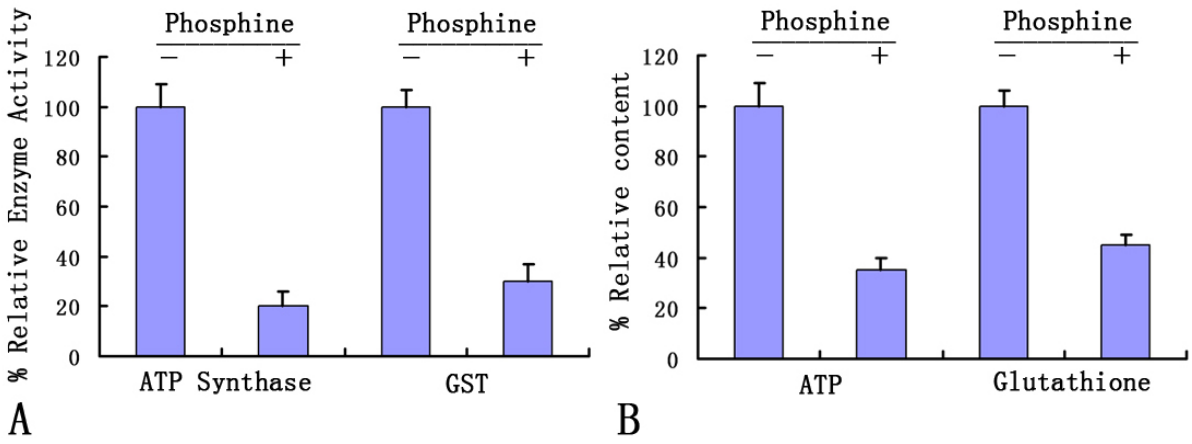

Figure 4. Enzyme activity assay results for treated insects. A The relative enzyme activities of ATP synthase and GST. B The relative contents of ATP and glutathione. Phosphine (–) refers to untreated insects, and phosphine (+) refers to insects treated with 330 ppm of phosphine for 24 h.

the cellular membrane (29,30). Epidermal growth factor-like repeats and discoidin I-like domains 3, supposedly transcription factors, are involved in tumor growth and embryonic development (31,32). Non-receptor type tyrosine-protein phosphatase, a tyrosine-protein phosphatase sublass, is a signaling molecule that regulates various cellular processes including cell growth, differentiation, and mitotic cycles (33). Our results, combined with that of other reports, reveal that phosphine regulates the expression of many proteins. Therefore, these six identified proteins may be used to transfer phosphine signals to target genes.

Lastly, all three proteins involved in defense were up-regulated. Phage shock protein significantly functions in the competition for survival under nutrient or energy limited conditions. Hence, the up-regulation of this protein may protect the moth from phosphine-induced ATP depletion (34,35). The DNA mismatch repair protein Msh2 could bind to DNA mismatches, thereby initiating DNA repair. This protein may be involved in the defense against phosphine-induced oxidative DNA damage (36,37). Ribosomal P0 protein is involved in the defense against malaria parasites, and in the selectivity of antifungal sordarin derivatives (38,39).

In conclusion, we identified the differentially expressed proteins in phosphine-treated peach fruit moth larvae. Our results indicate that phosphine causes complex transcriptional regulations that require further research.

6. ACKNOWLEDGMENT

This research was funded by the scientific research fund of the Chinese Academy of Inspection and Quarantine (No.2011JK012).

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Key Words: Phosphine, Peach fruit moth, Proteomics, Differential protein expression profiles

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