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Research Article

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Differentially Expressed Proteins From the Peritrophic Membrane Related to the Lethal, Synergistic Mechanisms Observed in *Hyphantria cunea* Larvae Treated With a Mixture of Bt and Chlorbenzuron

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Abstract

Hyphantria cunea (Drury) (Lepidoptera: Arctiidae) is an important forest insect pest around the world. It attacks a variety of broad-leaf trees. It has caused serious economic and ecological damage to its new habitats. A mixture of Bt and chlorbenzuron has a higher toxicity and faster killed than those of either agent alone to the 4th instar larvae of *H. cunea* both by the lab and field test results, and the toxic effect of the mixture treatment was significantly enhanced. Using proteomics technology, including SDS-PAGE and MALDI-TOF-TOF MS, we analyzed differentially expressed proteins of the peritrophic membrane (PM) of the 4th instar larvae of *H. cunea*, which were treated with the mixture. We identified 91 significantly differentially expressed proteins of the PM of the 4th instar larvae of *H. cunea* and those proteins were found to be involved in different metabolic pathways and processes. The energy-related and structural proteins made up the largest proportion of all of the identified proteins, and the mixture treatment of proteins was the small proportion of the identified structural proteins and energy-related proteins among the Bt, chlorbenzuron, and mixture treatments. Based on the proteomic data, we found that some proteins and their corresponding functions and pathways were related to the lethal mechanisms observed in 4th instar larvae of *H. cunea* when treated by the mixture.

Key words: a mixture of Bt and chlorbenzuron, expressed proteins, *Hyphantria cunea*, proteomics, the lethal mechanisms, the peritrophic membrane

The fall webworm *Hyphantria cunea* (Drury) (Lepidoptera: Arctiidae) is native to the USA and is an important forest insect pest around the world (Wei and Yao 2005). It is an invasive forest pest in China. It attacks a variety of plants, including many tree species and crops, especially broad-leaf trees growing on sidewalks and the sides of roads or on the edges of forestlands. It has caused serious economic and ecological damage to its new habitats. Therefore, it has been listed as a quarantine agent by many countries, including China (Wei and Yao 2005). *H. cunea* had entered China over 30 years ago and found causing damage in the northern Jiangsu since 2010 (Xu et al. 2013, 2015a, b). Many studies have focused on predicting and preventing the occurrence of this invasive insect; the areas of research on this species include its adaptability, sex pheromones, choice of hosts and natural enemies (Wei et al. 2003). However, studies on this invasive insect using proteomics remain scarce.

The peritrophic membrane (PM), a semi-permeable mucous layer, serves as a lubricating and protective barrier in the midgut of most types of insects. The PM is mainly composed of chitin and protein, and the latter is very important in maintaining the PM's dense structure (Zhang et al. 2009). Damage to PM proteins may cause serious interference to insect growth and may even lead to the death of young-instar larvae (Zhang et al. 2009). Thus, the study of PM proteins in different conditions of the 4th instar larvae of *H. cunea* will help to explain the lethal mechanisms of the mixture of Bt and chlorbenzuron.

A mixture of *Bacillus thuringiensis* (Bt) and chlorbenzuron has a high toxicity to the 4th instar larvae of the second and third generations of *H. cunea* (Xu et al. 2013). In addition, the mixture has killed the 4th instar larvae of *H. cunea* much faster than the single dose, and led to significantly enhanced toxic effect of the mixture treatment It has been concluded that the mixture of Bt and

chlorbenzuron is an effective and safe treatment for controlling *H. cunea* (Xu et al. 2013, 2015a, b).

The goal of this study was to apply proteomics technology, including two-dimensional electrophoresis (2-DE), MALDI-TOF-TOF MS, and MASCOT to compare the protein expression differences in the PM of the 4th instar larvae of *H. cunea* in relation to the synergistic lethal mechanisms in *H. cunea* larvae when the *H. cunea* larvae were exposed to a mixture of Bt and chlorbenzuron.

Materials

Source of the Tested Insects

The 4th instar larvae of *H. cunea* were collected from *Populus euramericana* cv. I-72 forest in XinYi City and reared with the leaves of *P. euramericana* cv. I-72 and *Acer negundo* L. in the forest pest inspection and quarantine laboratory in XinYi City of Jiangsu Province, China.

Sources of the Mixtures

The single dose of 8000 IU μL^{-1} Bt (Yangzhou Lüyuan Limited Co.) and 25% chlorbenzuron (Anyang Ruizhe Limited Co.) were used in this study. Bt was diluted to 0.1 IU μL^{-1} and chlorbenzuron was diluted to 0.313 $\mu L\ L^{-1}$. The mixture of Bt and chlorbenzuron was diluted to 0.05 and 0.156 $\mu L\ L^{-1}$, respectively, using distilled water. Distilled water was used as CK (Xu et al. 2015b).

Preparation of PMs

Fourth instar larvae of *H. cunea* were placed in 4°C pre-cooled 0.7% saline water larval abdomens were removed with a pair of pliers. Midguts were dissected with a scalpel from one end of the midgut which was gently torn to expose food parcels in the PM. The PM was washed with physiological saline water to remove food particles using a syringe until the PM became colorless and transparent (healthy larvae PM is colorless and transparent). The PMs were stored in a freezer at −20°C for further experiments.

Methods

Infection

Three hundred 4th instar larvae of *H. cunea* were used in this experiment, and six larvae were counted as one group. The experiment had four treatments: BT, C, M, and CK and each had three replicates. First, 12 groups were labeled as BT groups, 12 groups as C, 12 groups as M, and 12 groups as CK. A total of 48 groups were used. Then, the larvae of the BT groups were treated with Bt; the larvae of C, with chlorbenzuron; the larvae of M with a mixture of Bt and chlorbenzuron. Six, 12, 24, and 36 h after all larvae of each treatment were treated, three groups of larvae were sampled from each treatment to obtain PMs, and the PMs were immediately preserved in liquid nitrogen. The PM samples of the three groups from each treatment were mixed and pooled together, reducing the groups from 48 to 16 groups. The remaining 12 larvae were spared.

Protein Extraction and 2-DE

The PMs were extracted via TCA/acetone precipitation. The steps were as follows: the PMs of every group were ground in liquid nitrogen into a powder. The protein powder was put into a 50-ml centrifuge tube with 10% w/v TCA/acetone containing 0.1% DTT and held at −20°C for 2 h. The protein sample was centrifuged at 10,000 rpm for 15 min. The sample was then put in 10% w/v TCA/acetone containing 1 mM PMSF and 0.07% w/v β-mercaptoethanol and held in a freezer at −20°C for 1 h. After rinsing, the dried protein powder was dissolved in 800 µl of lysis solution containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM DTT, 1 mM PMSF and 0.5% v/v biolytes. In the process of centrifugation, insoluble materials were removed. The protein concentration of the sample was quantified according to the Bradford method (Bradford 1976).

Approximately 480 µg of proteins was separated by loading the sample on a 24-cm (pH 4–7) nonlinear gradient IPG strip (GE Healthcare), and electrophoresis was performed at 20°C for a total of 120,000 VH. The second electrophoretic dimension was performed using 12% SDS-PAGE. The signal was visualized by $AgNO_3$. Gel images were digitalized with a gel scanner (GS-800; Bio-Rad). Then, the gel image was analyzed with the PDQuest software package (Version 7.1.0; Bio-Rad).

With the assistance of the software guide, spots were detected, matched, and normalized according to the total density of the gels based on the parameter of percent volume. To determine the protein spots with different expression values, the abundance differences among the treatments (BT, C, M, CK) and the particular sampling times (6, 12, 24, and 36 h) were analyzed and compared. For each spot, the mean relative volume (RV) at every stage was computed. If the RV changed by more than 2.0-fold or less than 0.5-fold ($P < 0.05$), it was considered to be a differentially expressed protein.

In-gel Digestion and MALDI-TOF-TOF Analysis

All protein spots that were considered as differentially expressed proteins were manually excised from gels, washed with Milli-Q water three times, destained with 30 mM $K_3Fe(CN)_6$ in 50% 100 mM $Na_2S_2O_3$ and 100 mM NH_4HCO_3, dried twice with 100% acetonitrile, and digested overnight at 37°C with sequencing-grade modified trypsin (Promega, Madison, WI, USA) in 50 mM NH_4HCO_3. The final concentration of trypsin was 10 ng.μl^{-1}. The peptides were extracted twice with 0.1% TFA in 50% acetonitrile. Extracts were pooled and lyophilized. The resulting lyophilized tryptic peptides were dissolved in 5 mg ml^{-1} CHCA containing 0.1% TFA and 50% acetonitrile. MALDI-TOF-TOF MS (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometer) analyses were conducted with an UltrafleXtreme MALDI-TOF-TOF instrument (Bruker Daltonics Inc., USA). The target plate for the sample was an MTP AnchorChip 384 BC. The mass range was 700–3,500 in positive reflection mode for PMF, and 40–1015 for LIFT.

All spectra of differentially expressed proteins were submitted to a database search using the online MASCOT program (http://www.matrixscience.com) and compared against the NCBInr databases. The searching parameters were as follows: a 0.15-Da mass tolerance for peptides and a 0.25-Da mass tolerance for TOF-TOF fragments, which allowed trypsin miscleavage, were carbamidomethyl of Cys as a fixed modification, and the oxidation of Met and pyro-Glu formation of the N-terminal Gln and Glu as variable modifications. Only significant hits, as defined by the MASCOT probability analysis ($P < 0.05$), were accepted (Ma et al. 2012).

The proteins of the PM samples were divided into the functional classes according to the Kyoto Encyclopedia of Genes and Genomes (KEGG: http://www.kegg.jp/kegg/pathway.html) database and other relevant literature sources. In addition, differentially expressed proteins in the groups treated with BT, C and M were counted, and a Venn diagram was produced.

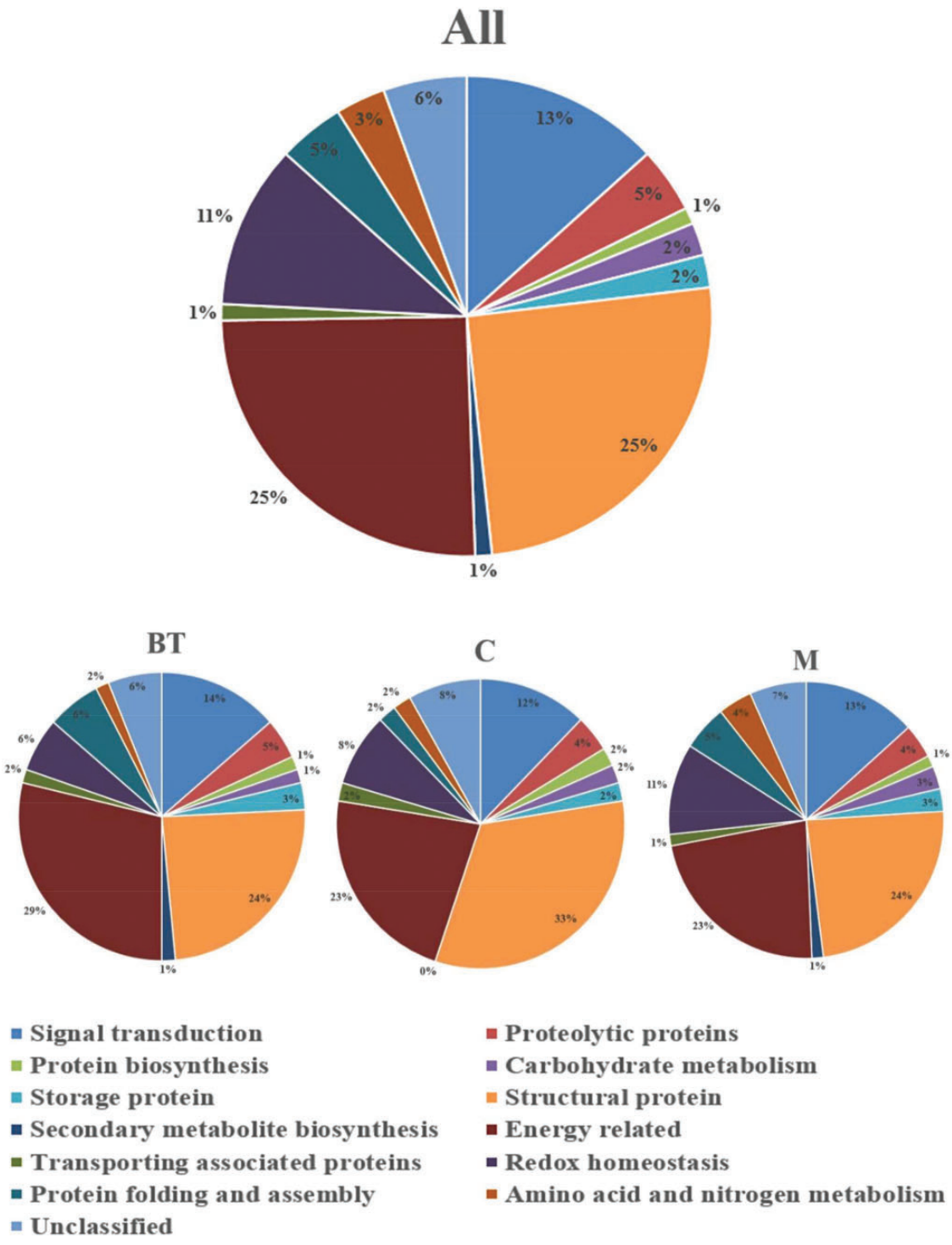

Fig. 1. Functional groups of the 91 differentially expressed and identified proteins of the PM of the larvae of *H. cunea*. Functional groups of the 91 differentially expressed and identified proteins in the separate treatment groups are marked as BT, C, M. Functional groups of differentially expressed and identified proteins in the BT, C, and M treatments. This classification is based on the KEGG (http://www.kegg.jp/kegg/pathway.html) database and other literature sources.

Results

2-DE Analysis and Identification of Differentially Expressed Proteins

2-DE maps were obtained using isoelectric focusing (IEF) on 24-cm (pH 4–7) nonlinear gradient (IPG) gels followed by SDS-PAGE on 12% polyacrylamide gels. Each 2-DE gel was stained with 0.1% $AgNO_3$. Within the pH range of 4–7 and the relative molecular mass range of 15–120 kDa, the 2-DE profile showed ~800 protein spots that could be reproducibly detected by PDQuest 7.1.0 software (Fig. 1). With the help of PDQuest 7.1.0 software, a total of 113 protein spots were obtained that had a more than 2.0-fold or less than 0.5-fold difference ($P < 0.05$) in expression values. The 113

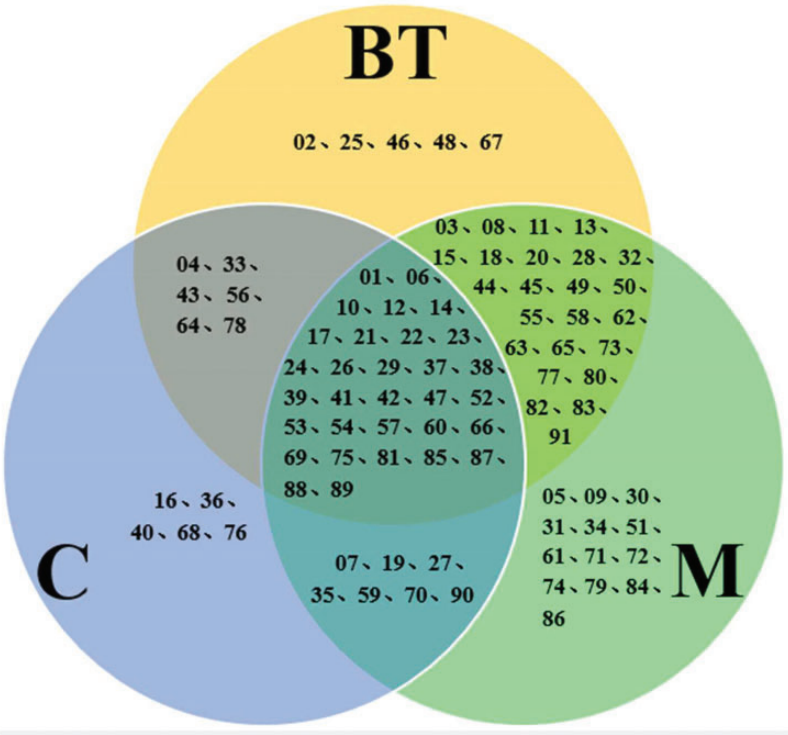

Fig. 2. Venn diagram of the 91 differentially expressed and identified proteins shown in Supp Table 1 [online only] of the PM of the larvae of *H. cunea*. Uppercase BT, C, and M indicate the treatment groups.

spots from the 2-DE gels were excised, extracted by in-gel digestion with trypsin, and subjected to mass spectrometry analysis. After the analysis, 91 proteins (Supp 1 and 2 [online only]) of the PMs of the 4th instar larvae of *H. cunea* were confidently identified according to the NCBI database. Their MASCOT search results and RVs are shown in Supp 1 [online only].

The 91 identified proteins were found to be involved in different metabolic pathways and processes, and they can be classified into the following functional groups: signal transduction (13%), proteolytic proteins (5%), protein biosynthesis (1%), carbohydrate metabolism (2%), storage proteins (2%), structural proteins (25%), secondary metabolite biosynthesis (1%), energy-related proteins (25%), transport-associated proteins (1%), redox homeostasis (11%), protein folding and assembly (5%), amino acid and nitrogen metabolism (3%), and unclassified (6%) in all content of proteins. The 'unclassified' proteins were those that successfully matched proteins in database with unknown functions and were usually unnamed protein products (Fig. 1). As shown in Fig. 1, the energy-related and structural proteins made up the largest proportion of the identified proteins. Proteins of C treatment were the highest proportion of the identified structural proteins among the Bt (24%), C (33%), and M (24%) treatments. Proteins of Bt treatment were the largest proportion of the identified energy-related proteins among the Bt (29%), C (23%), and M (23%) treatments. Proteins of M treatment was the highest proportion of the identified redox homeostasis and amino acid and nitrogen metabolism among the Bt (6%; 2%), C (8%; 2%), and M (11%; 4%) treatments. The rest of proteins were not significantly different from the proportion of the identified proteins among the Bt, C, and M treatments (Figs. 1 and 2).

Through the calculation of protein point expression of the PM samples, compared with the single dosage, the up-regulated protein spots were 15; down-regulated protein spots were 22, and up–down-regulated protein spots were 11 after treating with the mixture of Bt & chlorbenzuron. Differential expression proteins were further identified by mass spectrometry. The results indicated that treating with the mixture of Bt & chlorbenzuron could induce the stress and defense-related proteins to express; enhance structural proteins, signal transduction and energy-related protein; reduce energy-related protein, redox homeostasis and structural proteins; up–down-regulated structural proteins, energy-related proteins and protein folding and assembly, etc., in the PM samples. These changes are the protein base of defense reaction induced by treating with the mixture of Bt & chlorbenzuron in Fig. 3.

The Proteins Involved in Signal Transduction

In this study, 12 proteins of the PM were identified as being involved in signal transduction. Of those 12 proteins, 3 (spot 03, spot 05, spot 06) were identified as members of the 14–3–3 protein zeta group (Aitken 2006; Cheng et al. 2010). Three 14–3–3 proteins were up-regulated in general. This means that when the 4th instar larvae of *H. cunea* were treated with Bt, chlorbenzuron, or the mixture of Bt and chlorbenzuron, those signal transduction pathways were stimulated. There were three proteins identified as annexin proteins (spot 02, spot 08, and spot 11). The annexins are a family of Ca^{2+} lipid-binding proteins, which exist widely under the cytomembrane and near the organelles storing Ca^{2+}, as well as in the cell's nucleus or extracellular matrix. The expression of three annexin proteins was shown to be changed in this study. This suggests that when the PM was treated, the rate of apoptosis was altered (Fig. 2; Supp 2[online only]).

In addition to the 14–3–3 and annexin proteins, there were proteins related to troponin T (spot 01, spot 10), a protein similar to

Fig. 3. The expression trend of proteins related to pathway of glycolysis. Note: 1. The group of CK was marked in green color; group BT was marked in red color; group C was marked in yellow, and group M was marked in blue. 2. Red numbers was significant difference, and black numbers was no difference. The meaning of the numbers "46, 48, 49. . ." in Supp.2 are protein spots of 46, 48, 49. . ..

reticulocalbin (spot 04), sarcoplasmic calcium-binding proteins (spot 07, spot 12), and a type III effector protein (spot 09). Troponin T is a regulatory protein, which is involved in the control of calcium movement in muscle contraction. The protein similar to reticulocalbin and the sarcoplasmic calcium-binding proteins were related to cellular calcium signal transduction and Ca^{2+} binding (Fig. 2; Supp 2 [online only]).

Conclusions and Discussion

The results show that the mixture has killed the 4th instar larvae of *H. cunea* faster than the single dose, and led to significantly enhanced toxic effect of the mixture treatment. To research the lethal effects observed in the 4th instar larvae of *H. cunea* when treated by Bt, chlorbenzuron, and the mixture of both, we performed a comparative proteomic analysis of PMs of the 4th instar larvae of *H. cunea*. Larvae from the different treatment groups (BT, C, M, and CK) had their PM proteins extracted at various time points following the treatments. One hundred thirteen protein spots on the 2-DE gel out of the ~800 reproducible spots were differentially expressed and were successfully identified by MALDI-TOF-TOF MS analysis. The 91 identified proteins of the PM of the larvae of *H. cunea* were divided into 13 functional classes. Based on the proteomic data, we identified some of these proteins and their corresponding functions and pathways, which were related to the lethal effects of the treatment agents on the 4th instar larvae of *H. cunea*. This study will provide guidance and a theoretical basis for the integrated management of *H. cunea*, as well as for research on the pathogenic mechanisms of the treatment agents.

The Structural Proteins

In this study, 23 proteins of the PM were identified as structural proteins. They included actin, tropomyosin, tubulin, myosin, and paramyosin. Actin (spots 22, 27–32, 40, 42–44) is an important cytoskeletal protein and is involved in cell division, movement, migration, morphology, growth, and the maintenance of a variety of important physiological activities. The expression of actin was reduced in general. However, there were exceptions and increased expression was observed as follows: spot 28 (M treatment, 6 h), spot 31 (M treatment, 12 h and 36 h), spot 40 (C treatment, 12 h), spot 42 (BT treatment, 24 h), and spot 43 (BT treatment, 36 h and C treatment, 6 h). This suggests when the larval PMs of *H. cunea* were treated, the cellular structure of the larval PM was broken and supports the use of this treatment to kill the larvae of *H. cunea*. The tropomyosin (spots 23–25) proteins are binding proteins that interact with actin in thin filaments. The tubulin (spots 26, 34, and 38) proteins are involved in the formation of microtubules, which are part of the cytoskeleton. The myosin (spots 33, 35, 37, 39, and 41) and paramyosin (spot 36) proteins are the main components of muscle. Proteins of C treatment were the highest proportion of the identified structural proteins among the Bt (24%), C (33%), and M (24%) treatments. The small proportion of the identified structural proteins of Bt and M treatments may increase the damage of the structural proteins of the larvae of *H. cunea*. The changes in the expression of these proteins were consistent with death after the larvae of *H. cunea* were treated.

The Expression Trend of Proteins Related to Pathway of Glycolysis and the Energy-Related Proteins

The expression trend of proteins related to pathway of glycolysis was shown in (Supp.2 and Supp 3[online only]). Protein GPD-1 identified from spots 53 and 56 was up-regulated after group BT, group C and group M were treated in the early stages compared with CK, but down-regulated in the later stages. Protein GPD-1 controlled the central tache of carbohydrate metabolism, and its up-expression indicated that carbohydrate metabolism of the larvae of *H. cunea* were enhanced after treatments. This may be an emergency

reaction of the larvae of *H. cunea* suffered from adverse environment. Spot 60, which was identified as fructose 1,6-bisphosphate aldolase (ALD), catalyzed fructose-1,6-bisphosphate that resolved into G3P and DHAP. This reaction was reversible. The down-expression of ALD after group BT, group C, and group M treatments suggested that physiological function of the larvae of *H. cunea* were inhibited after treatments. Expression of triosephosphate isomerase, which was identified from spots 52, 57, 61, 62, and 64, was up-regulated in spot 57, but down-regulated in spots 52 and 61. Triosephosphate isomerase which was indispensable for generation of energy plays an important role in the pathway of glycolysis (Supp.2 and 3). The disorders of expression from triosephosphate isomerase showed that the physiology of the larvae of *H. cunea* was significantly influenced by the treatments. The last group of spots (46, 48, 49, and 55) was enolase, which decreased their expression after group BT, group C, and group M treatments. The expression pattern of enolase was the same as GAPDH. Since physiological function of the larvae of *H. cunea* was gradually weakened after the treatments, its energy metabolism was weakened, resulting in the death of larvae of *H. cunea*.

Proteins of Bt treatment were the largest proportion of the identified energy-related proteins among the Bt (29%), C (23%), and M (23%) treatments. The small proportion of the identified the energy-related proteins of C and M treatments may increase the damage of the energy-related proteins of the larvae of *H. Cunea*. In addition to the proteins mentioned above, ATP synthase (spots 47, 58, 65, and 66) and arginine kinase (AK) (spots 50, 51, 54, 63, 67, and 68) were identified. ATP synthase is a ubiquitous membrane enzyme that plays a key role in biological energy metabolism (Su et al. 2011). The expression of ATP synthase first increased and then reduced. It did not appear to follow a discernible pattern, which might be related to an overall decrease in energy metabolism. In invertebrates, AK plays a critical role in energy metabolism. AK is known to exist widely in invertebrates, providing energy for the activities of life and maintaining the ATP balance *in vivo* (Su et al. 2011). The expression of three AK proteins increased in this experiment. At the same time, the expression of three other AK proteins reduced. This is consistent with their role in maintaining the balance of ATP *in vivo* in the 4th instar larvae of *H. cunea*.

The Proteins Involved in Redox Homeostasis

Ten proteins of the larval PMs of *H. cunea* were identified as being involved in redox homeostasis (Liu et al. 2011). Hemocyanins (spots 71–73, 75, and 78) are copper-containing respiratory proteins in the hemolymph of many arthropods and mollusks. They are colorless when de-oxygenated, but turn light blue when oxygenated. The expression of hemocyanins was reduced except for the one indicated by spot 75 (C group, 36 h). This means that oxygen-carrying functions were weakened in the treated larvae. This reduction was associated with the lethal effects of the treatment agents on the larvae of *H. cunea*. Superoxide dismutase (SOD, spot 76) has a specific physiological activity and is the primary compound responsible for *in vivo* free radical scavenging. The level of SOD *in vivo* can be used as an indicator of the aging and death of the larvae of *H. cunea*. The expression of SOD was reduced, which meant that the free radical scavenging capacity of the larval PMs of *H. cunea* was decreased and aging and death were accelerated. Aldehyde dehydrogenase (spot 79) is responsible for the oxidation of aldehydes into their corresponding carboxylic acids. The expression of this enzyme increased. This was likely an emergency response by the larval PMs of *H. cunea* to the treatment with the lethal agents.

The Other Identified Proteins

In addition to the 68 proteins that have been described above, an additional 23 proteins of the PMs of the larvae of *H. cunea* were successfully identified, and their functions are discussed below.

In addition to the proteins and their corresponding functions and pathways related to the lethal effects of the treatment on the 4th instar larvae of *H. cunea* described above, other PM proteins of the larvae of *H. cunea* will be briefly discussed below.

Peroxiredoxin 2 (Prdx 2, spot 70) is one isoform of the peroxidases group. It distributed in the cytoplasm and plays an important role in protecting cells from oxidative damage (Herberts and De Frescheville 1981; Lang and van Holde 1991; Liu et al. 2011). As the expression of peroxiredoxin 2 was reduced, the ability of the larval PMs of *H. cunea* to protect cells from oxidative damage was reduced, which would accelerate cell death.

As the main protein component of the hemolymph, it typically represents up to 95% of the total protein content in penaeid shrimp (Herberts and De Frescheville 1981; Sellos et al. 1997). As a respiratory protein, its well-known role is to transport and store molecular oxygen (Redmond 1955). Its reduction means that the oxygen-carrying capacity was reduced in the larvae of *H. cunea*.

Glutathione *S*-transferase sigma (spots 74 and 77) plays an important role in the detoxification of insecticides in insects (Prapanthadara et al. 2000). This protein's expression was associated with the lethal effects of the agents on the larvae of *H. cunea*.

In addition to the 68 proteins described above, 23 proteins of the PM of the larvae of *H. cunea* were successfully identified and will be briefly discussed below.

Peptidase M13 (spot 13) can hydrolyze proteins to amino acids. The 25-kDa proteasome subunit (spot 14) and the 35-kDa proteasome subunit (spot 16) belong to proteasome group. Proteasomes are protein complexes found inside all eukaryotes and Archaea and in some bacteria (Peters et al. 1994). In eukaryotes, they are located in the nucleus and the cytoplasm (Peters et al. 1994). The main function of the proteasome is to degrade superfluous or damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds. The main function of the ubiquitin-like protein (spot 15) is to mark proteins for hydrolysis (Peters M. Raff, K. Roberts and J. D. Watson 1994). These four proteins are proteolytic proteins, and only the expression of the 25 kDa proteasome subunit increased, while the others reduced. This might mean after the larval PMs of *H. cunea* were treated with the agents, the activity of these proteins changed *in vivo*, and protein hydrolysis rates decreased.

Transposase (spot 17) is an enzyme that has an executive function in transposition, which has an important association with protein biosynthesis (Peters et al. 1994), and its expression was reduced. The reason is likely the same as with the above four proteolytic proteins.

The expression of pancreatic alpha-amylase (spot 18) was reduced. α-Amylase is an enzyme that hydrolyzes the alpha bonds of large, alpha-linked polysaccharides, such as starch and glycogen, yielding glucose and maltose (Maureen 2000). This means that the glucose levels were reduced *in vivo* in the larval PMs of *H. cunea*, which is an important cause of the decrease in energy metabolism. The expression of cathepsin L-like cysteine proteinase (spot 19) was different between different groups. Cysteine protease is a type of starch synthase, which mainly exists in the arteriolar wall and it produces amyloids (Lu et al. 2010). The activities of these enzymes might vary with changes in whole-organism metabolism.

Ferritin 2 (spots 20 and 21) serves to store iron in a non-toxic form, to deposit it in a safe form and to transport it to areas where it is required (Seckback 1982). Iron is involved in the formation of

hemoglobin and myoglobin and plays an important role in carrying oxygen and carbon dioxide (Seckback 1982). In this study, the expression of ferritin 2 increased at first and then decreased. This might be due to the fact that the emergency response increased oxygen transport when the larval PMs of *H. cunea* were treated. However, with the aging and death of the larvae of *H. cunea*, the metabolism began to slow down and to cease.

Cellular retinoic acid binding protein (spot 45) can promote optic retinoic acid metabolizing enzyme activities, enhancing the transformation rate of retinoic acid (Ma et al. 2008). In this study, the expression of this protein increased and decreased, which means that the transformation rate of retinoic acid changed.

The branched-chain amino acid ABC transporter substrate-binding protein (spot 69) had a decreased expression level in this study. The ABC transporter family is a large family of transmembrane proteins; their main function is to transport substrates out of the plasma membrane fueled by ATP hydrolysis (Ma et al. 2008). The decreased expression shows that the transmembrane transport capacity was weakened. It might relate to the change in metabolic rate.

The endoplasmic reticulum (ER) protein gp78 (spot 80) helps in proteins folding and assembly. Its expression in this study was decreased. This indicates that the folding and assembly rates of proteins were decreased in the ER. The 21.5-kDa heat shock protein (spots 81 and 82) is a member of the heat shock protein family with an alpha crystalline domain. Its main function is to maintain cytoskeletal stability, inhibit apoptosis, and to maintain the normal structure and function of cells under stress (Kappe et al. 2003; Golenhofen et al. 2004). In this study, the expression of this protein increased and then decreased, which might be a normal reaction after the larval PMs of *H. cunea* are treated. The T-complex protein 1 epsilon subunit (spot 83) acts as a molecular chaperone protein for tubulin and actin. All molecular chaperone families can help form biological macromolecules (mainly in protein folding and assembly functions) (Alberts et al. 1994). The expression of this protein increased at first and then decreased. This might be due to the emergency response and the increased aging and death of the larvae of *H. cunea*.

Glutamine synthetase 2 (spot 84) is an enzyme that catalyzes ammonia and glutamate binding to produce glutamine. Its increased expression indicates that nitrogen metabolism was increased. Translationally controlled tumor protein homolog (spot 85) can induce the cells to release histamine and has other functions that relate to processes, such as regulating inflammatory responses; anti-apoptosis effects; stress responses; regulating cell growth, proliferation and differentiation; and tumor reversion (Yan and He 2013). There were no observed patterns in this protein's expression that appeared to be specifically related to its multiple functions. Glutamine synthetase-like protein (spot 86) is similar to glutamine synthetase 2 (spot 84), but had a decreased expression.

In this study, there were five identified proteins in the PM with uncertain functions: unnamed protein products (spots 87, 89, and 91), uncharacterized protein LOC393586 (spot 88), and hypothetical protein DAPPUDRAFT_307177 (spot 90). Further study is needed to confirm their functions.

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