Proteomic-Based Approach to the Proteins Involved in 1-Deoxynojirimycin Accumulation in Silkworm *Bombyx mori* (Lepidoptera: Bombycidae)

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Abstract

1-Deoxynojirimycin (DNJ) is the most abundant poly-hydroxylated alkaloid in the latex of mulberry leaves and it protects mulberry from insect predation. However, silkworms can survive the poisoning effect of DNJ and accumulate DNJ by consumption of the mulberry leaves. In order to determine the molecular mechanism of DNJ accumulation in silkworm, comparative proteomic analysis was employed to evaluate protein expression in two groups of silkworm bodies (the third instar silkworm bodies had the maximum content of DNJ throughout life, and the newly hatched silkworm bodies had no DNJ). Our results indicated some differentially expressed proteins in the third instar silkworm involved in material metabolism, energy metabolism, oxidation-reduction, detoxification, immune, and transport regulation may correspond to the accumulation of DNJ. Furthermore, the expression level of five selected differentially expressed protein-encoding genes namely heat shock cognate protein (Hsp 70), glutathione S-transferase sigma 1 (GST), serine protease precursor (Ser), hemolymph protein (30K), and thiol peroxidredoxin (TPx) were investigated by quantitative real-time PCR and the accumulation of DNJ was measured by HPLC. Correlation analysis showed that the expression levels of Hsp70 and Ser were negatively correlated to DNJ accumulation with weak correlation, while 30K, GST, and TPx genes had positive correlation with DNJ accumulation. The findings suggested that these three proteins were probably important in the physiological process of DNJ accumulation in silkworm.

Key words: Silkworm (*Bombyx mori*), 1-Deoxynojirimycin (DNJ), Proteomics, two-dimensional electrophoresis, quantitative real-time PCR

Silkworm is the larva of *Bombyx mori* (Lepidoptera: Bombycidae), and it belongs to the order Lepidoptera. It is an insect with a significant importance in science as a model for molecular genetics, structural, and functional genomics studies as well as an economically valuable insect involved in the production of silk (Goldsmith et al. 2005, Mang et al. 2015). 1-Deoxynojirimycin (DNJ) is the most abundant poly-hydroxylated alkaloid present in the latex of mulberry leaves, and it protects mulberry from insect predation (Yoshiaki and Hivonu 1976, Asano et al. 2001, Konno et al. 2006). This poly-hydroxylated alkaloid is very toxic to other insect caterpillars that are not hosted by mulberry, such as castor mulberry and *Plutella xylostella* larvae; however, DNJ and its derivatives have no toxicity on silkworm that parasitizes on mulberry leaves. It has been reported that silkworm has developed some kind of defense mechanism against the toxicity and chemical defense of mulberry leaves as a result of coexistence (Yoshiaki and Hivonu 1976, Despres et al. 2007, Hirayama et al. 2007). DNJ content in the leaves of mulberry is found to be higher than other plants (Kim et al. 2003, Kimura et al. 2007, Yin et al. 2010) and silkworm larvae have the ability to accumulate DNJ with a special mechanism when they are reared on fresh young mulberry leaves (Asano et al. 2001, Liu et al. 2013, Chen et al. 2014). DNJ is a potent anti-diabetic, antiviral, and antioxidant agent (Hughes and Rudge 1994, Ryu et al. 1997, Pollock et al. 2008, Yatsunami et al. 2008). Nowadays, several products made from mulberry leaves and silkworm are very popular in South Korea, Japan, China, and other countries (Gui et al. 2004, Yang and Han 2006, Han et al. 2007, Lee et al. 2011, Lim et al. 2013).

However, there are very few reports on the molecular mechanism which has been adopted by silkworm in evading the chemical defense of mulberry thus achieving efficient enrichment of DNJ...
In order to study the mechanism of DNJ accumulation in silkworm and further use silkworm as an important bioreactor for the production of natural DNJ by regulating expression of relevant proteins, we previously examined the DNJ content at different life stages of silkworm and found the third instar silkworm bodies had the highest content of DNJ, and the newly hatched silkworm bodies had no DNJ (Liu et al. 2013). Therefore, in this study, the third instar silkworm and newly hatched silkworm bodies were chosen for the comparative proteomics analysis. Furthermore, we screened five differentially expressed proteins in the third instar silkworm body, which may possibly relate to DNJ accumulation, using quantitative real-time PCR (qRT-PCR) to detect the expression levels of selected protein-encoding genes and further analyzing their correlation to DNJ accumulation.

Materials and Methods
Silkworm Breeding and Sample Collection
Silkworm strains '7021' was obtained from the Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, China. Silkworms were reared under the conditions of temperature (25°C ± 1°C) and relative humidity (80% ± 2%). The silkworms were fed with the same fresh mulberry leaves (mulberry cultivar: Yu71-1 with 0.11% DNJ content, cultivated in mulberry field of Jiangsu University, Zhenjiang, China). The newly hatched and day 2 of the third instar silkworm bodies were collected, respectively, for the proteomics analysis. Besides, the silkworm bodies samples were collected in different life stages (from newly hatched to fourth instar) to detect the expression levels of selected genes, DNJ contents, and further correlation analysis. All silkworm bodies samples were starved for 24 h (except newly hatched silkworm bodies) and stored at −80°C until further use.

Protein Sample Preparation
All silkworm body samples were ground to powder in liquid nitrogen. Tris–HCl (pH 7.5) was used to extract proteins and was centrifuged at 15,000 rpm for 10 min. The supernatant was collected and precooled TCA/10% acetone (containing 0.07% (w/v) DTT) was added. The above mixture was precipitated for 2 h at 4°C and centrifuged at 15,000 rpm for 30 min. The pellet was washed twice with acetone (containing 0.07% (w/v) DTT), dissolved in lysis buffer (containing 7 M urea, 2 M thiourea, 4% (w/v) chaps, and 1% (w/v) DTT) and centrifuged at 15,000 rpm for 30 min. The supernatant used for protein analysis. The protein concentration was determined according to Bradford method (Bradford 1976).

Two-Dimensional Gel Electrophoresis Analysis (2-DE)
Isoelectric focusing (IEF) electrophoresis was performed with 17 cm (linear, pH 5–8) IPG strips at 20°C according to the manufacturer’s instruction (Ettan DALT six system, GE Healthcare) at a sample loading level of 300 µg. IEF was performed as follows: 250 V for 30 min, 1,000 V for 1 h, 8,000 V for 5 h, linear, 8,000 V for a total of 50,000 V/h, 500 V for 12 h. The strips were equilibrated in equilibration buffer I (6 mol/l urea, 50 mmol/l Tris-HCL, 0.07% (w/v) SDS, 30% (w/v) glycerol, and 1% (w/v) DTT) for 15 min and then in buffer II (replaced 1% DTT with 2.5% (w/v) iodoacetamide) for another 15 min. The equilibrated gel strip was subjected to 12.5% SDS–PAGE and sealed with 0.5% (w/v) agarose. SDS–PAGE was performed at a constant power of 100 V per gel for 30 min, and switched to 200 V per gel until the bromophenol blue frontier reached the bottom of the gel. The gels were stained with 0.1% (w/v) coomassie brilliant blue G-250 and scanned with a high-precision scanner (Scan Maker 9700XL) at a resolution of 600 dpi. Image analysis was performed using PD Quest 2D software. The experiment was done in triplicate for each sample.

In-Gel Digestion and Mass Spectrometry Analysis
Protein spots were excised manually and transferred into 1.5 ml RNase-free centrifuge tubes. The spots were washed three times with double distilled water, de-stained by sonication in 50 mM ammonium bicarbonate, 50% (v/v) acetonitrile, dehydrated with acetonitrile and dried in a vacuum pump. The dried proteins spots were treated by 10 mM DTT for 1 h at 56°C, alkylated with 55 mM iodoacetamide for 45 min at room temperature, washed with 25 mM ammonium bicarbonate and 50% (v/v) acetonitrile, respectively. It was further dehydrated with acetonitrile and incubated with 10–15 µl trypsin solution (Promega, 10 µg/ml) at 37°C overnight.

Fig. 1. 2-DE pattern of silkworm bodies proteins. The third instar silkworm bodies (A) and the newly hatched silkworm bodies (B). The identified differentially expressed protein spots in this study are indicated by arrows in the third instar silkworm bodies (A), the numbers corresponded to those are in Table 1.
| Spot No. | Protein name                                      | Accession No.     | pI/ molecular weight (kDa) | Coverage (%) | Score | Biological function          |
|---------|--------------------------------------------------|-------------------|--------------------------|--------------|-------|-------------------------------|
| 1       | HSC70                                            | gi|1495233 | 5.50/72.10          | 9            | 448                           | Molecular repair                     |
| 2       | Beta-1,3-glucan recognition protein 4 precursor  | gi|261245087 | 6.45/42.20          | 4            | 96                            | Signal transduction                  |
| 3       | Unknown                                          |                   |                          |              |      |                               |
| 4       | Vacuolar ATP synthase subunit B                 | gi|148298717 | 5.25/54.70          | 21           | 729                           | Energy metabolism                    |
| 5       | Cytosolic aspartate aminotransferase            | gi|220684   | 6.73/46.63          | 5            | 121                           | Detoxification                       |
| 6       | Vacuolar ATP synthase subunit B                 | gi|148298717 | 5.25/54.70          | 22           | 734                           | Energy metabolism                    |
| 7       | Heat shock cognate protein (Hsp70)              | gi|112982828 | 5.33/71.36          | 17           | 752                           | Molecular repair                      |
| 8       | Vacuolar ATP synthase catalytic subunit A       | gi|148298878 | 5.27/68.56          | 16           | 739                           | Energy metabolism                    |
| 9       | Vacuolar ATP synthase catalytic subunit A       | gi|148298878 | 5.27/68.56          | 16           | 704                           | Energy metabolism                    |
| 10      | Unknown                                          |                   |                          |              |      |                               |
| 11      | Vacuolar ATP synthase catalytic subunit A       | gi|148298878 | 5.27/68.56          | 16           | 762                           | Energy metabolism                    |
| 12      | Unknown                                          |                   |                          |              |      |                               |
| 13      | Cytosolic aspartate aminotransferase            | gi|220684   | 6.73/46.63          | 12           | 543                           | Detoxification                       |
| 14      | Vacuolar ATP synthase subunit B                 | gi|148298717 | 5.47/42.21          | 15           | 548                           | Energy metabolism                    |
| 15      | Vacuolar proton pump subunit B                  | gi|401326   | 5.26/55.10          | 21           | 659                           | Transportation                       |
| 16      | Vacuolar ATP synthase subunit B                 | gi|148298717 | 5.25/54.70          | 22           | 734                           | Energy metabolism                    |
| 17      | H+ transporting ATP synthase beta subunit        | gi|114052072 | 5.26/55.01          | 22           | 826                           | Energy metabolism                    |
| 18      | Glyceraldehyde-3-phosphate dehydrogenase         | gi|109119903 | 7.70/35.50          | 16           | 417                           | Energy metabolism                    |
| 19      | Unknown                                          |                   |                          |              |      |                               |
| 20      | Beta-1,3-glucan recognition protein 4 precursor  | gi|261245087 | 6.45/42.20          | 16           | 405                           | Signal transduction                  |
| 21      | Mitochondrial aldehyde dehydrogenase            | gi|114052408 | 7.52/56.21          | 4            | 72                            | Energy metabolism                    |
| 22      | Unknown                                          |                   |                          |              |      |                               |
| 23      | Elongation factor 1 gamma                        | gi|112983898 | 5.83/48.64          | 4            | 42                            | Cell function                        |
| 24      | Unknown                                          |                   |                          |              |      |                               |
| 25      | Arginine kinase                                  | gi|25453077 | 6.24/40.10          | 11           | 162                           | Energy metabolism                    |
| 26      | Translation elongation factor 2 isoform 1        | gi|112983010 | 6.23/98.20          | 7            | 387                           | Cell function                        |
| 27      | Translation elongation factor 2 isoform 1        | gi|112983010 | 6.23/98.20          | 3            | 184                           | Cell function                        |
| 28      | Keratin 1                                        | gi|7331218  | 8.16/66.15          | 4            | 204                           | Structure                           |
| 29      | Unknown                                          |                   |                          |              |      |                               |
| 30      | Unknown                                          |                   |                          |              |      |                               |

(continued)
The resulting peptides were collected and mixed with an equal volume of 10 mg/ml matrix solution (a-cyano-4-hydroxycinnamic acid), saturated with 0.1% (v/v) trifluoroacetic acid in 50% (v/v) acetonitrile, spotted on an MTP Anchor Chip (Bruker, Germany), and analyzed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS, Bruker, Germany) to acquire spectra with a mass range from 500 to 6,000 Da. External calibration was performed with standard peptides. The matrix and autolysis of trypsin were served as internal standards. The mass spectra were interpreted using the Mascot peptide mass fingerprint engine available on the website (http://www.matrixscience.com) for protein identification.

### Quantitative Real-Time PCR

All silkworm bodies (about 200 mg) for expression level analysis were ground with a mortar and pestle in liquid nitrogen. Total RNA was extracted using Trizol reagent (Invitrogen), subsequently, total RNA was reversely transcribed into cDNA using PrimeScriptTM RT reagent Kit (TaKaRa, RR047A). All the PCR reactions were carried out using SYBR Premix Ex TaqTM kit (TaKaRa, Kyoto, Japan) on a MX3000PTM system. Reaction system was 25.0 µl with 2.0 µl cDNA, 0.5 µl forward primer (20 mM), and 0.5 µl reverse primer. PCR amplification procedure was performed using the following program: 30 s at 95°C; 40 cycles of 5 s at 95°C, 20 s at 55°C; 40 s at 72°C. All samples were run in triplicate for each gene. The expression level of each gene was quantified relative to silkworm housekeeping gene (actin-3), and comparison of Ct value method was used to quantify gene expression level.

### HPLC Determination of DNJ Content in Silkworm Bodies From Different Life Stages

All silkworm bodies samples for DNJ analysis were lyophilized, smashed, and passed through a 80-mesh screen. Dried silkworm body powder of 0.1 g was taken and transferred into 1.5 ml centrifuge tube, 10 ml hydrochloric acid (0.05 M) was added and mixed evenly. The solution was centrifuged at 12,000 rpm for 15 min, and the supernatant was obtained. The residue was extracted one more time as above. The two extraction supernatants were merged in a...
and distilled water was added to a total volume of 50 ml, then the resulting extraction solution was made. Resulting extraction solution 10 µl was treated with 10 ml borate saline buffer (pH 8.5), 5 mM FMOCl solution (20 µl), mixed thoroughly and placed in 20°C water bath for 20 min, and subsequently, 10 µl of 0.1M glycine was added to quench the remaining FMOCl. Finally, 950 µl of 0.1% (v/v) acetic acid was added to stabilize the DNJ-FMOC formed in the reaction (Yin et al., 2010). The sample was analyzed for DNJ measurement with the aid of RP-HPLC. The HPLC analysis was performed with a JASCO LC-1500 instrument (JASCO, Japan) with a reversed-phase column HiQSiL C18 (5 µm, 250 mm × 4.6 mm). The column was eluted with a mobile phase of acetonitrile: 0.1% aqueous acetic acid (55:45, v/v) at 1.0 ml/min. Analysis was monitored using a UV detector (254 nm) throughout the entire run. A sample of 10 µl was injected to the column. A two-point external standard method was used to calculate the concentration of DNJ in silkworm samples. All samples were performed in triplicate.

Statistical Analysis
Data are presented as mean ± standard error of three independent experiments. Statistical analysis was carried out using SPSS version 16.0 software (SPSS Inc, Chicago, IL). One-way analysis of variance followed by Fisher’s post hoc analysis. Differences were considered statistically significant at P < 0.05.

Results and Discussion

Comparison of Protein Patterns
Determination of expressed proteins was achieved with the aid of 2-DE separation using Coomassie Blue G-250. Prominent differences were observed between the 2D-PAGE patterns of proteins from the third instar silkworm and newly hatched silkworm bodies. There were 961 individual protein spots detected in the third instar silkworms, the molecular weight ranged from 22 to 97 kDa, while the pl (isoelectric point) was from 5.0 to 8.0 and 1,060 protein spots were identified in newly hatched silkworm with a molecular weight range from 25 to 100 kDa, with pl range of 5.0–7.5. Out of these, 499 protein spots matched, with a matching ratio of 49.53% (Fig. 1A and B).

Mass Spectra Analysis
Eighty differentially expressed protein spots with high abundance from the third instar silkworms were excised from 2-DE gels and subjected to in-gel trypsin digestion and subsequent MALDI-TOF/TOF mass spectrometry. Out of these spots, 64 spots were successfully identified (Fig. 1A; Table 1). All identified proteins were classified into nine categories: 22 spots were related to energy metabolism (vacuolar ATP synthase subunit B, vacuolar ATP synthase catalytic subunit A, 34.38%); 11 spots for molecular repair (such as HSC70 and Hsps, 17.19%); seven spots for oxidation-reduction...
The result suggested that silkworm could probably accumulate DNJ by feeding on mulberry leaves through the processes of molecular repair, energy consumption, detoxification, autoimmunity, and oxidation resistance.

Expression Levels of Selected Protein-Encoding Genes, DNJ Accumulation, and Their Correlations

After analyzing and comparing all identified proteins functions (Table 1), we chose these five differentially expressed protein-encoding genes (Table 2) which may play a major role in DNJ accumulation process and investigated their expression levels related to DNJ accumulation. The five genes relative mRNA levels from newly hatched to fourth instar silkworm bodies were detected with the aid of qRT-PCR analysis. Silkworm actin-3 (Act-3) gene was used as housekeeping gene, and their primers were designed using Primer 5.0 software. The primer sequences were listed in Table 2. Simultaneously, DNJ content from newly hatched silkworm to fourth instar silkworm bodies was determined too. The changes observed in the expression levels of the five genes mentioned above and DNJ content were shown in Fig. 2, the analyzed correlations between DNJ content and expression levels of the five genes were shown in Fig. 3.

As shown in Fig. 2A and C, the relative expression levels of Hsp70 and Ser genes were observed to be significantly high in the first instar silkworm as compared to the second and third instar which had very low expression levels of these two genes. In contrast, there was a gradual increase in the relative expression of glutathione S-transferase sigma 1 (GST) and 30K genes from the newly hatched to the third instar. The third instar had the highest relative expression of these two genes, and a slight decline in the relative expression was observed for the fourth instar (Fig. 2B and D). As indicated in Fig. 2F, DNJ content was also observed to obviously change at different silkworm life stages. There was a gradual increase in DNJ content from the newly hatched to the third instar. The highest DNJ content was accumulated in the third instar stage of the silkworm. Correlation analysis also showed that there was a weak negative correlation between DNJ content and the expression level of genes Hsp70 and Ser (Fig. 3A and C), suggesting that these two genes might not play any significant role in DNJ enrichment. However, the relative expression levels of GST, 30K, and thiol peroxiredoxin
(TPx) genes had strong positive correlation to DNJ content (Fig. 3B, D, and E), which suggested that these three genes may be most likely involved in DNJ accumulation.

GST gene is one of the three enzymatic superfamilies which functions as a detoxifier (Liu et al. 2010), scavenge oxygen free radicals and catalyzes the conjugation of electrophilic compounds to glutathione, thus playing a critical role in cell survival and is an integral component of the antioxidant defense against reactive oxygen species (Hayes et al. 2005, Yamamoto et al. 2006, Yamamoto et al. 2009). It is also involved in intracellular transport, hormone synthesis, and protection of cell injury caused by oxidative stress (Board et al. 2000). The 30K gene is one of the essential energy storage proteins during the growth and development process of silkworm, and it plays important roles in the embryonic development of silkworm through the formation of vitellogenin and material transport (Ujita et al. 2002, Zhong et al. 2005). The 30K protein has also been reported to display a strong resistance to apoptosis (Yu et al. 2011). TPx, an antioxidant protein, functions in the removal of reactive oxygen free radicals. External stimulus and pathogenic micro-organisms infection usually induce TPx protective mechanism against their harmful effects. Previous reports have indicated that upon a viral infection, the silkworm will induce the production of TPx to reduce oxidative damage and enhance the activity of its natural killer cells (Lee et al. 2005).

DNJ, a potent glucosidase inhibitor, is an important defense mechanism for mulberry. Yet, silkworm can enrich itself with DNJ from mulberry leaves. Daimon et al. (2008) found out that the expression of β-fructosyltransferase in silkworm was abnormal and its activity was not influenced by DNJ, which partially explained the adaptation of silkworm to mulberry-defense mechanism. In this study, we further discovered that the expression levels of GST, 30K, and TPx genes specifically increased along with the enrichment of DNJ in different life stage of silkworm bodies. This indicated that these three genes in silkworm may play very important roles in the physiological process of DNJ accumulation to adapt to mulberry-defense mechanism. From this, we can reasonably extrapolate that DNJ enrichment in silkworm or its adaptation to mulberry chemical defense was a very complex physiological processes with a lot of proteins involved.

Conclusion

In this study, the third instar silkworm and the newly hatched silkworm bodies were used to evaluate protein expression, and 64 differentially expressed proteins spots were identified from the third instar silkworm bodies. Our result indicated that silkworm could probably accumulate DNJ by regulating the processes of substances metabolism, energy consumption, detoxification, autoimmunity, and oxidation resistance. The expression levels of five selected protein-encoding genes and their correlation to DNJ accumulation suggested that GST, 30K, and TPx genes were more likely involved in the process of DNJ accumulation. Thus, our findings provided a scientific basis for further study to reveal the mechanism of DNJ accumulation in silkworm and use silkworm as a bioreactor to produce natural DNJ by regulating the expression of genes related to DNJ accumulation.

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Author Contribution Statement

Z.O. conceived the experiments and provided the main experimental financial and technical support; H.C. and Y.L. carried out the experimental procedures; W.W. and H.C. performed qRT-PCR experiment; G.P. provided the silkworm variety “7021”, H.C. and O.J.O. performed data analysis and wrote the article. All authors approved the final version.

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