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Research Article

Wild Silkworm Cocoon Contains More Metabolites Than Domestic Silkworm Cocoon to Improve Its Protection

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Abstract

The silk of silkworm consists of fibroin fiber coated by sericins. In addition, some nonprotein components were also identified in the sericin fraction. The presence of nonprotein components in the silk has not been well explained. In the present study, methods based on gas chromatography–mass spectrometry were used to identify the metabolites in the cocoon silk from a wild silkworm and two domestic silkworm strains. In total, 45 metabolites were in the cocoon silk, including organic acids, fatty acids, carbohydrates, amino acids, and hydrocarbons. Comparative analyses revealed that 17 metabolites were significant more in the wild silkworm cocoon than in the domestic silkworm cocoon, including three organic acids, three fatty acids, three aldoses, four sugar alcohols, three hydrocarbons, and pyridine. Of them, citric acid in the wild silkworm cocoon is more than 40 times that in the domestic silkworm cocoon, which may have protective value against microbes. The carbohydrate, lipid, and the long-chain hydrocarbons may act as water repellent to make the pupa survive longer in the dry environment. Many metabolites in the cocoon silk may play roles to improve the silk resistance. Lots of nonprotein components were identified in the silk for the first time, providing useful data for understanding the biological function of the cocoon silk.

Key words: agricultural entomology, organic chemistry

The silk of silkworm consists of fibroin filaments coated by protective cover of sericins. Besides fibroins and sericins, other proteins were also identified in the silks, mainly including enzymes, seroins, protease inhibitors, and proteins of unknown functions (Dong et al. 2013, Zhang et al. 2015). In addition, silk also contains small amounts of nonprotein components, including 0.4–0.8% wax matter, 1.2–1.6% carbohydrates, 0.7% inorganic matter, and 0.2% pigment (Gulrajani et al. 1988), which are mainly associated with the sericin fraction (Mercer et al. 1954).

Many studies have focused on pigments of colored silks, and found that mulberry silkworm can spin white, yellow or yellow-green silk, and nonmulberry silkworms produce cocoons range in color from pale to dark brown (Bergmann et al. 1939a, Brunet et al. 1974). The green pigment results from flavonoids (Kurioka et al. 2002, Tamura et al. 2002), while the yellow coloring matter are carotenoids (Tabunoki et al. 2004; Sakudoh et al. 2007). Tanning agents are responsible for the brown color of silks, four of which have been identified, including DOPA, *N*-3,4-dihydroxyphenyllactyl DOPA, 3-hydroxyanthranilic acid glucoside and gentisic acid glucoside (Brunet et al. 1974; Kawasaki et al. 1985; Kramer et al. 1989, 1987; Przibram et al. 1927; Waite et al. 1992).

Previous studies found that silk wax of silkworm mainly contains higher primary alcohols, higher fatty acids, and long-chain normal hydrocarbons, the chain lengths of which were estimated as C_{25}–C_{32} (Bergmann et al. 1938). The silk wax were also found in bees, which comprises hydrocarbons, alcohols, esters, and other materials (Garnier et al. 2002, Kimpe et al. 2002). In the hornet *Vespa analis* (Hymenoptera: Vespidae), silk wax consisted mainly of hydrocarbons with linear carbon chain C_{24}–C_{34} (Kameda et al. 2007). The silk wax increases the silk resistance against the action of chemicals, as well as excessive humidity and desiccation (Bergmann et al. 1938).

We speculate that silk may contain various components that play protective roles against predation, infection, dehydration, and radiation. Fibroins and sericins build physical barrier to protect pupae, whereas protease inhibitors and seroins in the silk have antimicrobial activities (Dong et al. 2016, Guo et al. 2016, Singh et al. 2014). Some nonprotein silk components may also have protective roles. Carbohydrate, wax, and salt act as water repellents, flavonoids possess antimicrobial activity against a wide range of pathogens (Dzoyem et al. 2013), and other nonprotein components may be the metabolites of biochemical reactions in the silk gland. In fact, many enzymes, which are involved in metabolism of carbohydrates,

lipids, proteins, and other materials, have been identified in the silk gland and silk (Dong et al. 2013, 2016; Zhang et al. 2015); however, their roles in the silk have not been well explained. Studies on silk metabolites will provide important information to reveal the roles of enzymes.

In the current study, methods based on gas chromatography–mass spectrometry (GC/MS) were used to identify the metabolites in the cocoon silk from a wild silkworm and two domestic silkworm strains. Comparative analyses revealed that a number of metabolites displayed significantly higher abundance in the wild silkworm cocoon than in the domestic silkworm cocoon. Our results provide valuable data to reveal the nonprotein components in the silk and are also helpful for understanding the biological function of the cocoon.

Materials and Methods

Material and Sample Preparation

The two strains Dazao and Haoyue of domestic silkworm, *Bombyx mori*, were provided by the State Key Laboratory of Silkworm Genome Biology, Southwest University. The wild silkworm *B. mandarina* was collected from Changshou, Chongqing, China. The *B. mori* and *B. mandarina* were reared on mulberry leaves. The cocoons were collected and stored in microcentrifuge tubes at 4°C until required. The clean cocoons were weighed (0.01 g), cut into small pieces, and treated in 121°C water for 2 h. After centrifugation (14,000 × *g*, 15 min), the supernatant solution was transferred into a new centrifuge tube, and the precipitate was washed with 80% methanol. Incorporating the supernatant solution and the washing solution, add methanol to give a final methanol concentration of 80%. The mixture was left at 4°C overnight and then centrifuged at 14,000 × g for 15 min. The supernatant was lyophilized, and resuspended in 200 µl of 80% methanol.

GC-MS Analysis

The surpernatant was recovered by centrifugation (14,000 × g, 15 min, 4 °C). Surpernatant (100 µl) was lyophilized, and resuspended in 80 µl of methoxyamine solution (20 mg/ml in pyridine), and then was placed in water bath at 37°C for 1.5 h. Subsequently, 60 µl of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) was used for silylation for 1 h. The reaction was stopped by addition of 10 µl hexane. The analysis was performed using the platform based on a DB-5MS column (0.25 µm, 0.25 mm × 30 m, Agilent Technologies, Inc., Santa Clara, CA) and Agilent 7890B-5977A system (Agilent Technologies, Inc.). The temperatures of inlet and ion source were set at 280 and 230°C, respectively. The oven temperature program was set as following: initially kept at 60°C for 3 min, increased to 170°C at the rate of 5 °C/min, 4 °C/min to 234°C, 5°C/min to 280°C, and held for 5 min. The voltage of detector was set at 0.93 kV, and the EI ionization voltage of metabolites was 70 eV. Full scan mode (*m/z* 33–500) was applied to acquire mass signals.

Data Preprocessing and Analysis

Raw data files were firstly converted into NetCDF data format by Agilent ChemStation, and sequentially peak features were extracted by XCMS software running under R environment, version 2.3.1. All the peak areas of metabolites were normalized to the total area. The metabolites detected by GC-MS were identified through searching against the National Institute of Standards and Technology (NIST) library.

To show the separated trend of sample sets, the PLS-DA (partial least squares-discriminant analysis) was performed using SIMCA-P software (version 11.0; Umetrics). The KEGG IDs of metabolites were obtained by searching the KEGG pathway database (http://www.kegg.jp/kegg/pathway.html) (Kanehisa et al. 2000, 2014). The classification and pathway of metabolites were built by using the KEGG IDs. Unparied *t*-test with *P*-value <0.05 were used to compare the difference in metabolite abundance between wild silkworm cocoon and domestic silkworm cocoon. The heat map was used to show the differential metabolites by using the HemI (Heatmap Illustrator, version 1.0.3.3) (Deng et al. 2014).

Results

Metabolites in the Silkworm Cocoon

B. mori was believed to have evolved from *B. mandarina*. The size and color of *B. mori* cocoon have been influenced during long-term domestication. The wild silkworm cocoon is small and has brown-yellow pigment as protective color, the cocoon of domestic silkworm Dazao has medium size and is yellow-green, and the cocoon of domestic silkworm Haoyue is big and white (Fig. 1A). GC-MS analysis was performed to identify the metabolites in the silkworm cocoons. In total, 45 metabolites were identified by GC-MS (Suppl Table 1 [online only]). Cocoon metabolites were classified into six categories: organic acid, fatty acid, carbohydrate, amino acid, hydrocarbon, and other metabolites (Suppl Table 1 [online only]). Many metabolites were reported in this species for the first time.

The eight identified organic acids contain acetic acid, butanoic acid, benzoic acid, ethanedioic acid, propanoic acid, citric acid, succinic acid, and butenedioic acid (Suppl Table 1 [online only]). Of them, citric acid, butanedioic acid (succinic acid), and butenedioic acid (maleic acid and fumaric acid) are involved in TCA cycle. Four fatty acids were tetradecanoic acid (14: 0), hexadecanoic acid (16: 0), octadecanoic acid (18: 0), and 9,12,15-octadecatrienoic acid (18: 3). Eight carbohydrates could be divided into aldose (d-galactose, d-mannose, d-lyxose, d-allose, and arabinose), sugar alcohol (glycerol, myo-Inositol, xylitol, mannitol), sugar acid (gluconic acid), and amino sugar (*N*-acetyl-d-glucosamine). Four amino acids, glycine, alanine, serine, and proline, are the main constituent amino acids of silk proteins. Among the nine hydrocarbons, including propene, dodecane, tridecane, tetradecane, octadecane, heneicosane, hexacosane, heptacosane, and octacosane, only propene is alkene, others are alkanes. Other metabolites in the cocoon include urea, threonic acid, ribonic acid, 2-thiobarbituric acid, acetamide, pyridine, and piperidine.

Differential Cocoon Metabolites Between *B. Mandarina* and *B. Mori*

The total ion chromatograms suggested that metabolites in the three kinds of cocoons have obvious differences (Fig. 1B). PLS-DA showed that data points were clearly clustered into three distinct groups, suggesting that the cocoon components were different among the *B. mandarina* and two *B. mori* strains (Fig. 1C). By comparing the relative levels of metabolites across samples, 32 metabolites displayed significantly different levels between two sample groups ($P < 0.05$; Fig. 2 and Suppl Table 1 [online only]). In total, 17 metabolites had significant higher levels in the *B. mandarina* cocoons than in the *B. mori* cocoons (Fig. 3), including three organic acids, three fatty acids, three aldoses, four sugar alcohols, three hydrocarbons, and pyridine. It is interesting that citric acid in the wild silkworm

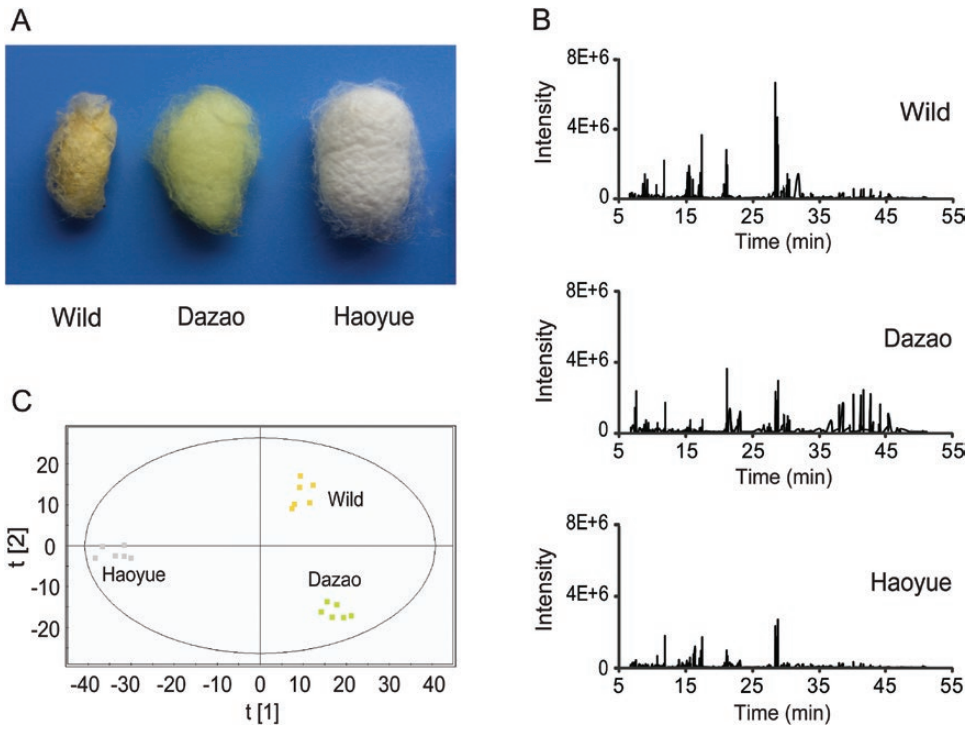

Fig. 1. GC-MS-based metabolic profiles of cocoons from the wild silkworm and two domestic silkworm strains, Dazao and Haoyue. (A) Photos of cocoons from the wild silkworm and two domestic silkworm strains. (B) GC-MS-based metabolic profiles of the wild silkworm cocoon and domestic silkworm cocoon. (C)The score plot of PLS-DA analysis of GC-MS-based metabolic profiles of the wild silkworm cocoon and domestic silkworm cocoon.

cocoon is more than 40 times that in the domestic silkworm cocoon (Fig. 3 and Suppl Table 1 [online only]). Three metabolites (arabinose, threonic acid, and ribonic acid) are more in the Dazao than in the wild silkworm and Haoyue, whereas no metabolities have relative higher level in the Haoyue than in wild silkworm and Dazao (Suppl Table 1 [online only]).

Pathway Analysis of the Cocoon Metabolites

Revealing the pathways is helpful for understanding the generation processes of types of metabolites. The identified metabolites in this study were submitted to the KEGG database to obtain the compound IDs. The obtained 35 compounds were then submitted to map the pathways. One hundred and nine pathways were identified, which were involved in the metabolisms of carbohydrates, amino acids, fatty acids, and other substances. By integrating these pathways, we found that many carbohydrates could transform into each other, such as the transformations among allose, mannitol, fructose, and manose (Fig. 4). It is noted that *N*-acetyl-d-glucosamine (GlcNAc) is the degraded product of chitin, whereas GlcNAc could be reused to generate chitin (Fig. 4). Although pyruvate and acetyl-CoA were not identified in this study, they are important intermediate metabolites to link carbohydrate metabolisms, amino acid metabolisms, organic acid metabolisms, fatty acid metabolisms, and hydrocarbon metabolisms (Fig. 4).

Discussion

The silk of silkworm has been used over millennia for textiles, and there is an increasing interest on the properties and application of silk in recent years. Materials scientists revealed that silk formed cocoon has well-designed structure and excellent mechanical properties to resist impact forces generated by predators (Zhao et al. 2005, 2007; Blossman-Myer et al. 2010; Pandiarajan et al. 2011; Chen et al. 2012a, b, 2013), while biologist found that silk contains varies antimicrobial components to protect inside pupae against infection (Akai et al. 1997; Nirmala et al. 2001; Korayem et al. 2007; Shaik et al. 2009; Dong et al. 2013; Singh et al. 2014; Zhang et al. 2015). In addition, the silk was also thought to play protective roles against dehydration and radiation. It is very important to reveal the silk components for understanding the purpose of silk-spinning and expanding the application field of silk. This study identified various organic acid, fatty acid, carbohydrate, amino acid, hydrocarbon, and other metabolites from the silk, and compared their abundance difference between wild silkworm and domestic silkworm, and discussed their biological functions in the silk gland and cocoon.

There are 523 and 182 enzymes in the silk gland and silk, respectively (Dong et al. 2013; Dong et al. 2016), implying that lots of intermediates or products resulting from metabolisms could be detected in the silk. Hydrolases consist of 50% enzymes in the silk, mainly including glycosidases, lipases, and proteases. In addition, 61 binding and transport proteins in the silk have potential abilities to interact with carbohydrates, lipids, and other small molecules. These results jointly pointed out that carbohydrates, fatty acids, and amino acids should be important metabolities in the silk. In fact, 11 carbohydrates, four fatty acids, and four amino acids were found in the silk in the present study. It is noted that glycine, alanine, and serine are the main constituent amino acid of silk proteins, according for 45, 29, and 12% in the fibroins, and 17, 5, and 29% in the sericins (Mondal et al. 2007).

Many small molecules showed significantly higher abundance in the wild silkworm than in the domestic silkworm, such as

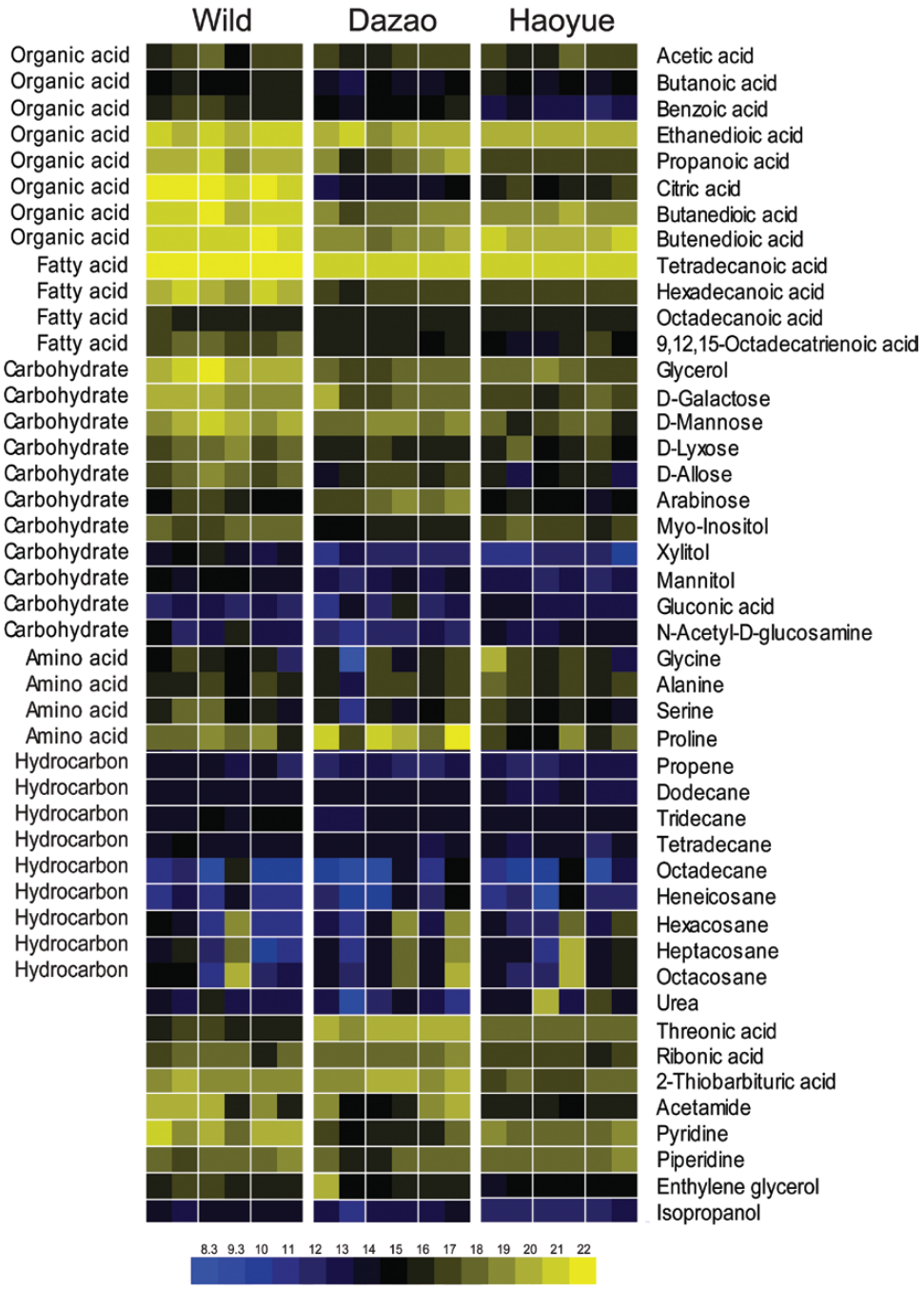

Fig. 2. Heat map of abundances for the silk metabolites from the wild silkworm and two domestic silkworm strains. This heat map was generated using Heml. The abundance of metabolites is represented by different colors from yellow (higher) to blue (lower). The classification of compounds was showed on the left, and the compound names were showed on the right.

carbohydrates, organic acids, fatty acids, and hydrocarbons. Three organic acids, including citric acid, succinic acid, and fumaric acid, are involved in the tricarboxylic acid cycle. They are relatively more in the wild silkworm cocoon than in the domestic silkworm cocoon, reflecting that the metabolism is very exuberant in the silk gland of wild silkworm. These metabolites in the wild silkworm cocoon could strengthen its protection on the pupa. For example, organic acids have antibacterial activity (Cherrington et al. 1991), and carbohydrates and lipids may act as the water repellent. The long-chain hydrocarbons are waxes, which could provide water-retaining property for the cocoon (Bergmann et al. 1938, 1939a, b), and make the pupa survive longer in the dry environment.

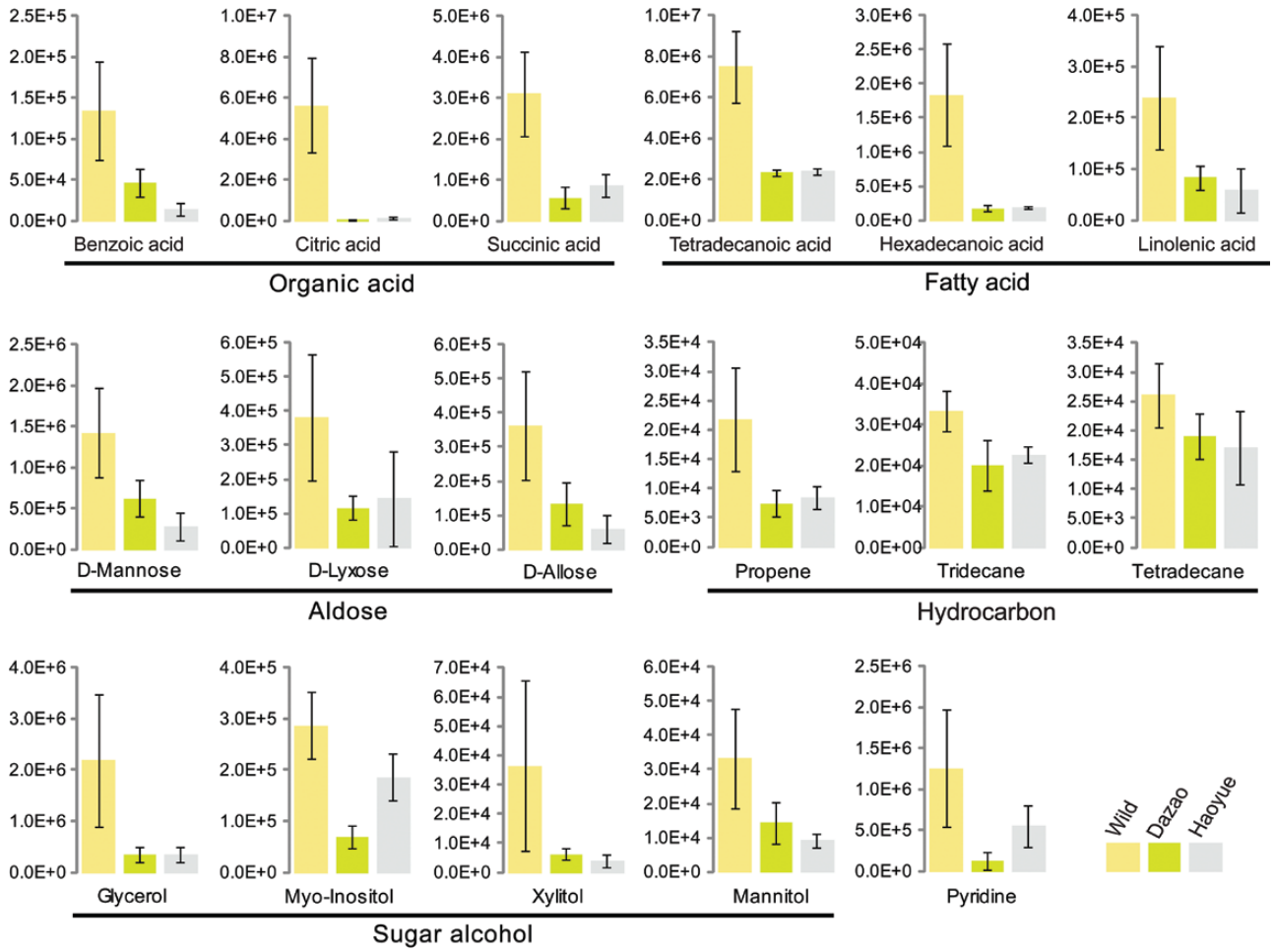

Fig. 3. Seventeen silk metabolites showed significant higher levels ($P < 0.05$) in the wild silkworm cocoon than in the domestic silkworm cocoon. The *y*-axis represents the compound abundance, which is the value of the peak area computed by the software XCMS.

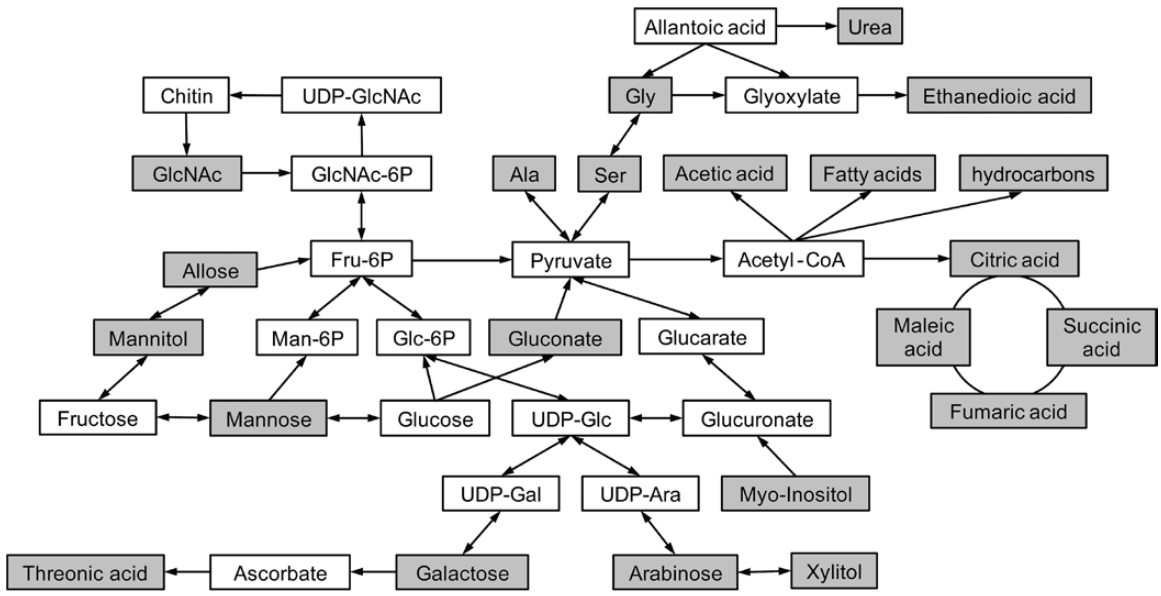

Fig. 4. Metabolic pathways of the silk metabolites involving in the carbohydrates, fatty acids, amino acids, organic acids, and hydrocarbons. The grey boxes represent the identified metabolites in this study.

Acknowledgments

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Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

References Cited

Akai, H. 1997. Anti-bacteria function of natural silk materials. Int. J. Wild Silkmoth Silk. 3: 79–81.

Bergmann, W. 1938a. A study of the composition of the ether soluble fraction of raw silk. Text. Res. J. 8: 221–225.

Bergmann, W. 1938b. The distribution of wax in cocoon silk. Text. Res. J. 8: 195–199.

Bergmann, W. 1939a. The natural pigments of silk. Text. Res. J. 9: 397–408.

Bergmann, W. 1939b. Silk wax and certain physical properties of raw silk. Text. Res. J. 9: 175–182.

Blossman-Myer, B., and W. W. Burggren. 2010. The silk cocoon of the silkworm, bombyx mori: macro structure and its influence on transmural diffusion of oxygen and water vapor. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 155: 259–263.

Brunet, P. C., and B. C. Coles. 1974. Tanned silks. Proc. R. Soc. Lond. B. Biol. Sci 187: 133–170.

Chen, F., D. Porter, and F. Vollrath. 2012a. Silk cocoon (*Bombyx mori*): multi-layer structure and mechanical properties. Acta Biomater 8: 2620–2627.

Chen, F., D. Porter, and F. Vollrath. 2012b. Structure and physical properties of silkworm cocoons. J. R. Soc. Interface 9: 2299–2308.

Chen, F., T. Hesselberg, D. Porter, and F. Vollrath. 2013. The impact behaviour of silk cocoons. J. Exp. Biol. 216: 2648–2657.

Cherrington, C. A., M. Hinton, G. C. Mead, and I. Chopra. 1991. Organic acids: chemistry, antibacterial activity and practical applications. Adv. Microb. Physiol 32: 87–108.

Dong, Z., P. Zhao, C. Wang, Y. Zhang, J. Chen, X. Wang, Y. Lin, and Q. Xia. 2013. Comparative proteomics reveal diverse functions and dynamic changes of bombyx mori silk proteins spun from different development stages. J. Proteome Res 12: 5213–5222.

Deng, W., Y. Wang, Z. Liu, H. Cheng, and Y. Xue. 2014. Hemi: a toolkit for illustrating heatmaps. PLoS One 9: e111988.

Dong, Z., Q. Song, Y. Zhang, S. Chen, X. Zhang, P. Zhao, and Q. Xia. 2016. Structure, evolution, and expression of antimicrobial silk proteins, seroins in lepidoptera. Insect Biochem. Mol. Biol. 75: 24–31.

Dong, Z., P. Zhao, Y. Zhang, Q. Song, X. Zhang, P. Guo, D. Wang, and Q. Xia. 2016. Analysis of proteome dynamics inside the silk gland lumen of bombyx mori. Sci. Rep 6: 21158.

Dzoyem, J. P., H. Hamamoto, B. Ngameni, B. T. Ngadjui, and K. Sekimizu. 2013. Antimicrobial action mechanism of flavonoids from dorstenia species. Drug Discov. Ther 7: 66–72.

Garnier, N., C. Cren-Olivé, C. Rolando, and M. Regert. 2002. Characterization of archaeological beeswax by electron ionization and electrospray ionization mass spectrometry. Anal. Chem 74: 4868–4877.

Gulrajani, ML. 1988. Degumming of silk; in Silk dyeing printing and finishing. Department of Textile Technology Indian Institute of Technology, New Delhi, 63–95.

Guo, X., Z. Dong, Y. Zhang, Y. Li, H. Liu, Q. Xia, and P. Zhao. 2016. Proteins in the cocoon of silkworm inhibit the growth of Beauveria bassiana. PLoS One 11: e151764.

Kameda, T., T. Akino, and K. Kojima. 2007. Wax components of larval cocoon silk of the hornet vespa analis fabricius. Anal. Bioanal. Chem 387: 2895–2902.

Kanehisa, M., and S. Goto. 2000. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28: 27–30.

Kanehisa, M., S. Goto, Y. Sato, M. Kawashima, M. Furumichi, and M. Tanabe. 2014. Data, information, knowledge and principle: back to metabolism in KEGG. Nucleic Acids Res 42: D199–D205.

Kawasaki, H, and H. Sato. 1985. The tanning agent in the silk of the Japanese giant silkmoth, *Dictyoploca japonica* butler. Insect Biochem. 15: 681–684.

Kimpe, K., P. A. Jacobs, and M. Waelkens. 2002. Mass spectrometric methods prove the use of beeswax and ruminant fat in late roman cooking pots. J. Chromatogr. A. 968: 151–160.

Korayem, A. M., T. Hauling, C. Lesch, M. Fabbri, M. Lindgren, O. Loseva, O. Schmidt, M. S. Dushay, and U. Theopold. 2007. Evidence for an immune function of lepidopteran silk proteins. Biochem. Biophys. Res. Commun. 352: 317–322.

Kramer, K. J., and T. L. Hopkins. 1987. Tyrosine metabolism for insect cuticle tanning. Arch Insect Biochem. 6: 279–301.

Kramer, K. J., V. Bork, J. Schaefer, T. D. Morgan, and T. L. Hopkins. 1989. Solid state 13C NMR and chemical analysis of insect noncuticular support structures: mantid oothecae and cocoon silks. Insect Biochem. 19: 69–77.

Kurioka, A., and M. Yamazaki. 2002. Purification and identification of flavonoids from the yellow green cocoon shell (sasamayu) of the silkworm, *Bombyx mori*. Biosci. Biotechnol. Biochem. 66: 1396–1399.

Mercer, E. H. 1954. Studies on the soluble proteins of the silk gland of the silkworm, *Bombyx mori*. Text Res J. 24: 135–145.

Mondal, M, K. Trivedy, and S. N. Kumar. 2007. The silk proteins, sericin and fibroin in silkworm, *Bombyx mori* Linn. – a review. Caspian J. Env. Sci. 5: 63–76.

Nirmala, X., D. Kodrík, M. Zurovec, and F. Sehnal. 2001. Insect silk contains both a kunitz-type and a unique kazal-type proteinase inhibitor. Eur. J. Biochem. 268: 2064–2073.

Pandiarajan, J., B. P. Cathrin, T. Pratheep, and M. Krishnan. 2011. Defense role of the cocoon in the silk worm *Bombyx mori* L. Rapid Commun. Mass Spectrom. 25: 3203–3206.

Przibram, H., and H. Schmalfuss. 1927. Das Dioxyphenylalanin in den Kokons des Nachtpfauenauges *Samia cecropia* L. (Saturnidae). Biochem Zeitschr 187: 467–469.

Sakudoh T., H. Sezutsu, T. Nakashima, I. Kobayashi, H. Fujimoto, K. Uchino, Y. Banno, H. Iwano, H. Maekawa, T. Tamura, et al. 2007. Carotenoid silk coloration is controlled by a carotenoid-binding protein, a product of the Yellow blood gene. Proc Natl Acad Sci USA. 104: 8941–8946.

Shaik, H. A., and F. Sehnal. 2009. Hemolin expression in the silk glands of *Galleria mellonella* in response to bacterial challenge and prior to cell disintegration. J. Insect Physiol. 55: 781–787.

Singh, C. P., R. L. Vaishna, A. Kakkar, K. P. Arunkumar, and J. Nagaraju. 2014. Characterization of antiviral and antibacterial activity of *Bombyx mori* seroin proteins. Cell. Microbiol. 16: 1354–1365.

Tabunoki, H., S. Higurashi, O. Ninagi, H. Fujii, Y. Banno, M. Nozaki, M. Kitajima, N. Miura, S. Atsumi, K. Tsuchida, et al. 2004. A carotenoid-binding protein (CBP) plays a crucial role in cocoon pigmentation of silkworm (*Bombyx mori*) larvae. FEBS Lett. 567: 175–178.

Tamura, Y., K. Nakajima, K. Nagayasu, and C. Takabayashi. 2002. Flavonoid 5-glucosides from the cocoon shell of the silkworm, *Bombyx mori*. Phytochemistry 59: 275–278.

Waite, J. H. 1992. The DOPA ephemera: a recurrent motif in invertebrates. Biol. Bull 183: 178–184.

Zhang, Y., P. Zhao, Z. Dong, D. Wang, P. Guo, X. Guo, Q. Song, W. Zhang, and Q. Xia. 2015. Comparative proteome analysis of multi-layer cocoon of the silkworm, *Bombyx mori*. PLoS One 10: e123403.

Zhao, H., X. Feng, S. Yu, W. Cui, and F. Zou. 2005. Mechanical properties of silkworm cocoons. Polymer 46: 9192–9201

Zhao, H., X. Feng, W. Cui, and F. Zou. 2007. Mechanical properties of silkworm cocoon pelades. Eng Fract Mech 74: 1953–1962.