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GC/MS-based metabolomic studies reveal key roles of glycine in regulating silk synthesis in silkworm, *Bombyx mori*

Quanmei Chen [a, 1], Xinyu Liu [b, 1], Ping Zhao [a], Yanhui Sun [a], Xinjie Zhao [b], Ying Xiong [a], Guowang Xu [b, **], Qingyou Xia [a, *]

[a] State Key Laboratory of Silkworm Genome Biology, Southwest University, 216 Tiansheng Road, Chongqing 400715, China
[b] Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China

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ABSTRACT

Metabolic profiling of silkworm, especially the factors that affect silk synthesis at the metabolic level, is little known. Herein, metabolomic method based on gas chromatography-mass spectrometry was applied to identify key metabolic changes in silk synthesis deficient silkworms. Forty-six differential metabolites were identified in *Nd* group with the defect of silk synthesis. Significant changes in the levels of glycine and uric acid (up-regulation), carbohydrates and free fatty acids (down-regulation) were observed. The further metabolomics of silk synthesis deficient silkworms by decreasing silk proteins synthesis using knocking out fibroin heavy chain gene or extirpating silk glands operation showed that the changes of the metabolites were almost consistent with those of the *Nd* group. Furthermore, the increased silk yields by supplying more glycine or its related metabolite confirmed that glycine is a key metabolite to regulate silk synthesis. These findings provide important insights into the regulation between metabolic profiling and silk synthesis.

1. Introduction

Silks, produced by arthropods (e.g., silkworm, spider), are a class of proteinaceous materials with superior mechanical properties, including high biocompatibility, good biodegradability, and lacks of immunogenicity and allergenicity. The silk materials have been widely used in many fields, such as biomaterial, medicine, nutrient (Altman et al., 2003; Leal-Egana and Scheibel, 2010; Lee et al., 2011; Meinel et al., 2006; Numata et al., 2011; Sofia et al., 2001; Tao et al., 2012; Yucel et al., 2014). For example, spider silks can be applied to guide biomaterial for human neurons (Roloff et al., 2014). Silks from silkworm have been used as biomedical sutures and in textile production for many years. It was advantageous that silkworms can be reared in captivity, the properties of its silk have been well characterized, however, the regulation of silk synthesis at metabolic levels is not well known.

The silkworm, *Bombyx mori*, is an important insect economically and was domesticated over the past 5000 years from the wild progenitor *Bombyx mandarina*. Silk glands are the specialized organs for producing silk proteins, and then silk proteins are stored in the lumen of silk gland and spun to generate silk. Silks are used to produce cocoon shell for protecting the pupa. The silk protein components mainly comprise fibroins and sericins (Gamo et al., 1977). Fibroins, which consist of fibroin heavy chain (fib-H), fibroin light chain (fib-L) and P25, are secreted from the posterior silk gland cells (Yamaguchi et al., 1989). Sericins, which mainly include sericin-1, sericin-2 and sericin-3, are secreted from the middle silk gland cells (Takasu et al., 2007). Previous studies reported that several transcriptional factors are involved in transcriptional regulation of silk protein genes in silkworm. For example, silk gland factor-1 (SGF-1) is involved in regulation the serecin-1 gene (Mach et al., 1995); The silk gland-specific factor

Abberviations: fib-H, fibroin heavy chain; GC–MS, gas chromatography-mass spectrometry; FGKO, fibroin heavy chain gene knocked-out; SGE, silk glands extirpation; ZFN, zinc finger nuclease; EI, electron impact; *m/z*, mass-to-charge; RT, retention time; QCs, control samples; NIST, National Institute of Standards and Technology; RSD, relative standard deviation; PCA, Principal component analysis; PLS-DA, partial least squares – discriminant analysis; ACPC, 1-aminocyclopropane carboxylic acid.

* Corresponding author. Tel.: +86 023 68250099.
** Corresponding author. Tel./fax: +86 411 84379530.
E-mail addresses: xugw@dicp.ac.cn (G. Xu), xiaqy@swu.edu.cn (Q. Xia).
[1] These two authors contributed equally to this work.

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SGFB and an ubiquitous factor BMFA are involved in the tissue-specific expression and temporal control of P25 gene, respectively (Nony et al., 1995); The helix-loop-helix transcription factor *Bmsage* is involved in regulation of *fib-H* gene (Zhao et al., 2014). However, to our best knowledge, the research on the regulatory factors in silk synthesis at the metabolic level is very rare up to date. Although both genomic and proteomic techniques have been widely utilized in various silkworm researches (Dong et al., 2013; Yi et al., 2013; Zhao et al., 2012, 2010), metabolomic technique have not been applied to the study of silkworms. As the downstream of both genomics and proteomics, the metabolomics is urgently required to apply in silkworm studies.

Metabolomics is utilized for the study of the dynamic response of small molecules in biological systems under different conditions. It is a powerful tool not only for nutrition research (Brennan, 2013), but also for exploring potential biomarkers and therapeutic targets for diseases (Huang et al., 2013; Zhang et al., 2011). Importantly, metabolomic analysis can provide breakthroughs in mechanistic research (Wu et al., 2012; Xu et al., 2012), and new insights into biological response mechanisms (Barding et al., 2013; D'Alessandro et al., 2013; Obata and Fernie, 2012). Therefore, the metabolomic method would be applied to explore the regulation of silk synthesis in silkworm.

To address the regulation of silk synthesis in silkworms at metabolic level, the hemolymph of silkworms were studied by using metabolomic method based on gas chromatography-mass spectrometry (GC–MS). The experimental materials were obtained through the following three ways. One group is *Nd* group, including the mutant silkworm strain *Nd* with deficient silk glands, and silkworm strain *dazao* and *21-872* with normal silk glands. One is FGKO group that the silk synthesis was artificially arrested by knocking out the *fib-H* gene and its control. And the third one is SGE group, including the silkworms with extirpated silk glands, and the control of sham operations (SO) and untreated silkworms. The results showed that the levels of glycine and urea acid were substantially up-regulated, and the levels of carbohydrates, free fatty acid and some amino acids were down-regulated in the hemolymph of all selected silk synthesis deficient silkworms. Interestingly, the production of silk synthesis was increased by injecting glycine and its related metabolite ACPC (1-aminocyclopropane carboxylic acid). Hence, those results demonstrated that glycine is a key regulatory factor in the silk synthesis.

2. Materials and methods

2.1. Experimental insects and treatments

The silkworm strains *dazao*, *naked pupa* (*Nd*) and *21-872* were gained from the silkworm stock pool of the Southwest University of China. In consideration of that the developmental stage of fifth instar larvae is a vital life stage for silkworms, and the hemolymph can reflect the global metabolic status. Hence, the hemolymph of three silkworm stains from the third day to the sixth day in the fifth instar larvae (five biological replicates of each point) was collected and then analyzed using GC–MS. Given that the *fib-H* protein encoded by *fib-H* gene is the main component of silk protein, and silk glands are the organs for producing silk proteins. Therefore, *fib-H* gene was knocked-out and silk glands were extirpated for further study. The *fib-H* gene was knocked-out using ZFN (zinc finger nuclease) technology in *dazao* silkworms. All the details for knocking out *fib-H* gene were described in reference (Ma et al., 2014). Hemolymph samples with 20 biological replicates for *fib-H* gene knocked-out and 19 biological replicates for its control were used. The silk glands extirpation (SGE) were performed on the second day of fifth instar larvae. To better compare the changes of metabolites after extirpating silk glands, sham operations (SO) were also performed as the SEG, except for the removal of the silk glands. The silk glands (both left and right) were extirpated on ice using forceps on the dorsal surface incision performed by using surgical scissors. 10 biological replicate samples were collected from SGE, SO, and normal, respectively. Hemolymph samples from all selected silkworms were collected on ice and stored at −80 °C immediately. Three female larvae were used for each biological replicate. Silkworms were reared on mulberry leaves at 26 ± 1 °C.

2.2. Sample preparation for GC–MS analysis

To remove proteins, all hemolymph samples selected in this work (80 μL) were precipitated with methanol (320 μL), in which tridecylic acid (10 μg/mL) was pre-added as internal standard. And then, 80 μL of methoxyamine solution (20 mg/mL in pyridine) was used to dissolve the lyophilized residues. It was placed in water bath at 37 °C for 1.5 h oximation reaction, followed by 15 min' ultrasound. Subsequently, 65 μL of MSTFA (N-methyl-N-(trimethylsilyl) trifluoroacetamide) was used for silylation in the same environment conditions for 1 h. Ultimately, after 10 min' centrifugation at 13,000 rpm, the supernatant was transferred for subsequent GC–MS analysis.

2.3. Instrument parameters for data acquisition

Metabolomic analysis of *Nd*, *dazao* and *21-872* based on GC–MS was performed by using Agilent 7890-5975C system (Agilent Technologies, Palo Alto, CA), and that of FGKO group and SGE group was carried out on Shimadzu QP2010 GC–MS system (Shimadzu Corporation, Kyoto, Japan) equipped with EI (electron impact) ionization source. To obtain good separation, the DB-5MS column (0.25 μm, 0.25 mm × 30 m, Agilent Technologies, Inc, Santa Clara, CA) was used. The temperatures of inlet, transfer interface and ion source were set at 300, 280, and 230 °C, respectively. The oven temperature program was set as following: initially kept at 70 °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 270 °C, then ramped to 300 °C at 10 °C/min, 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*: 33–600) was applied to acquire mass signals.

2.4. Metabolomic data preprocessing

Raw data files were firstly converted into NetCDF data format by Agilent ChemStation, and sequentially *m/z* features matched by XCMS software running under R environment version 2.3.1. For the purpose of avoiding information redundance, the metabolic features were firstly identified by comparing their mass spectra with the NIST 2011, version 2.0 (National Institute of Standards and Technology, USA) library and standard compounds. Then the areas of identified metabolites were extracted by Agilent ChemStation or Shimadzu GC–MS solution workstation, respectively. And all metabolites were normalized to total area before univariate and multivariate analysis. Validation of the analytical method, including the reproducibility, intra-day and inter-day precision, and stability of post-preparation, was evaluated by the percentage of relative standard deviation (RSD %) of all detected metabolites of quality control samples (QCs). QC samples were prepared by adding the equal-volume of all selected biological replicates from each group samples.

2.5. Data analysis

To obtain the separated trend of sample sets, PCA (principal component analysis) and PLS-DA (partial least squares-