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Comparative proteomic analysis of the silkworm middle silk gland reveals the importance of ribosome biogenesis in silk protein production

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ABSTRACT

The silkworm middle silk gland (MSG) is the sericin synthesis and secretion unique sub-organ. The molecular mechanisms of regulating MSG protein synthesis are largely unknown. Here, we performed shotgun proteomic analysis on the three MSG subsections: the anterior (MSG-A), middle (MSG-M), and posterior (MSG-P) regions. The results showed that more strongly expressed proteins in the MSG-A were involved in multiple processes, such as silk gland development and silk protein protection. The proteins that were highly expressed in the MSG-M were enriched in the ribosome pathway. MSG-P proteins with stronger expression were mainly involved in the oxidative phosphorylation and citrate cycle pathways. These results suggest that the MSG-M is the most active region in the sericin synthesis. Furthermore, comparing the proteome of the MSG with the posterior silk gland (PSG) revealed that the specific and highly expressed proteins in the MSG were primarily involved in the ribosome and aminoacyl-tRNA biosynthesis pathways. These results indicate that silk protein synthesis is much more active as a result of the enhancement of translation-related pathways in the MSG. These results also suggest that enhancing ribosome biogenesis is important to the efficient synthesis of silk proteins.

1. Introduction

The silk gland of the silkworm is worthy of study, as its prominent function of synthesizing silk protein in both basic and applied researches [1–4]. The silk gland is anatomically and physiologically divided into three subparts: the anterior silk gland (ASG), middle silk gland (MSG), and posterior silk gland (PSG). Each subpart has a unique function in cocoon formation. Silk fibers are mainly composed of the core protein fibroin and the coat proteins sericins, which are synthesized by the PSG and MSG, respectively. As a result, the MSG is more suitable for expressing exogenous proteins, as it is more practical to extract proteins from the sericin layer than the fibroin layer [2,4]. Considering the economic significance of silk production, understanding the molecular basis of MSG protein synthesis is highly important.

The MSG, with approximately 230 cells, develops as a one-cell layered glandular epithelium and is the unique sub-organ responsible for synthesis of sericin [5]. Sericins are a group of hydrophilic glue proteins mainly composed of sericins 1, 2 and 3 that surround the fibroin core and make up 20–30% of silk protein [5,6]. These sericin proteins are synthesized in different MSG subsections, including the anterior (MSG-A), middle (MSG-M), and posterior (MSG-P) regions [5]. The sericin genes, encoding glue proteins, are expressed specifically in the MSG with sub-organ localized specificity. The expression of *Ser1* is restricted to only the MSG-P in early larval instars, and expands to the MSG-M in the last instar [5,7,8]. Meanwhile, the *Ser2* and *Ser3* genes are expressed mainly in the MSG-A [5,7,9–11]. This spatio-temporal regulation of sericin gene expression in the three MSG regions suggests unique control of sericin synthesis in the MSG [10,12]. The expression of sericin genes is regulated by various factors. For example, *Ser1* has two binding sites, SA (around −90) and SC (around −200), in its promoter that stimulate its transcription in vitro [13,14]. Although *Bombyx* fork head (Fkh) protein and POU-homeodomain protein POU-M1 can bind to the SA and SC, respectively [15,16], POU-M1 can negatively regulate the expression of *Ser1* [8,17,18]. Besides, the Hox protein Antp, a component of the MSG–intermolt-specific complex (MIC), binds to the essential promoter element of *Ser1* and activates its expression [19].

In addition to the studies on the transcriptional regulation factors of sericin genes, large-scale expression profiling analyses of the silk gland have been carried out. Comparative analysis of MSG and PSG transcriptomes has shown that MSG cells have a wide spectrum of functions in addition to their major role in sericin synthesis and secretion [20,21]. Differential expression of proteins from different sections of the silk gland has been analyzed [22,23]. The PSG expression profiles at transcriptional, translational, and post-translational modification levels during the fifth instar have been characterized [24]. However, the proteome of the MSG is not well understood, and proteomic

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differences among the three subsections of the MSG have not been deeply analyzed. The molecular basis of the functional differences between the MSG and PSG is still not clear. In the present study, we analyzed the proteome of the three subparts of the MSG using shotgun approaches with label-free quantification. We also compared the protein expression between the three MSG regions, as well as between the MSG and PSG, with the aim of revealing the molecular basis of the biological functions of these regions.

2. Materials and methods

2.1. Silkworm tissue collection

Silkworm strain P50 was reared on fresh mulberry leaves under the standard conditions (25 °C, and 80% R.H.). The MSG was dissected in cold physiological saline at the third day of the fifth instar (V3). The whole MSG was divided into three subsections: MSG-A, —M, and -P. The silkworms were from a homozygous strain with high genetic similarities. We randomly selected 15 silkworm larvae and divided them into three groups as biological repeats for proteomic analysis. To avoid contamination by secreted sericins into the gland lumen, the MSG was immersed in pre-chilled 60% ethanol for 1 min to denature the sericin proteins, which were then drawn out from the MSG lumen with nippers. For gene expression analysis, RNase free tips and solutions were used for all steps of experiment.

2.2. Protein sample preparation and SDS-PAGE

Protein extraction from the MSG-A, —M, and -P was performed as described previously [24,25]. The extracted protein samples were quantified using the 2-D Quant Kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Totally 200 μg of proteins for each sample were separated by SDS-PAGE using a 12.5% resolving gel followed by Coomassie Brilliant Blue (CBB) staining.

2.3. Nano-LC–MS/MS analysis

The MSG proteins were separated by SDS-PAGE, and the gels were sliced into 12 sections followed by in-gel digestion and mass spectrometry (MS) analysis, according to our previously described methods [24,25]. Briefly, the digested peptide sample was re-suspended and subjected to an Ettan MDLC nanoflow/capillary LC system (GE Healthcare, Pittsburgh, PA) coupled to a linear ion trap Orbitrap mass spectrometer (LTQ-Orbitrap XL™, Thermo Fisher Scientific, Bremen, Germany). The LTQ-Orbitrap machine was operated with XCalibur software (version 2.0, Thermo Electron, San Jose, CA, USA). Collision-induced dissociation (CID) was controlled with normalized collision energy of 35%, and activation q of 0.25 for MS/MS acquisition. The five most intense ions were isolated for CID fragmentation and measured in the linear ion trap with the dynamic exclusion settings: repeat count 2, repeat duration 30 s, exclusion duration 180 s. Triplicate replicates were performed for each sample.

2.4. Protein identification

The retrieved MS/MS data was searched against the same database which was previously used, containing 1739 entries of silkworm protein sequences from NCBI Refseq and 14,623 entries of the predicted silkworm genome coding sequences [24]. The MS/MS data were automatically submitted to the in-house Mascot server for database search using Mascot Daemon software (version 2.2, Matrix Science, London, U.K.). The parameters for database searching were the same as in our previous study with minor changes [24]. Briefly, the parent and fragment ion mass tolerances were set at 50 ppm and 0.6 Da, respectively. Two missing cleavage sites were allowed for tryptic digestion. A fixed (carbamidomethyl) modification on cysteine and variable modifications on oxidation (M) were specified. To control for the false discovery rate (FDR), the resultant database search files were subjected to further processing by the Trans-Proteomic Pipeline (TPP, version 4.6) using PeptideProphet and ProteinProphet algorithms with the probability thresholds at 0.7 and 0.9, respectively [24,26]. The proteins identified with at least two assigned peptides were acceptable. To reduce the redundancy of the identifications, the proteins assigned in one group with common peptides were manually screened according to the previous method [24].

2.5. Label-free quantification

The relative expression levels of the proteins identified in the MSG-A, —M, and -P were evaluated by Absolute Protein Expression (APEX) scores [27]. All parameters were consistent with our previous report [24]. To compare differential protein expression between the MSG and PSG at V3 of the silkworm larvae, we analyzed the TPP processed data from the MSG (this study) and the PSG (previous research) [24] using the APEX analysis software [27]. The relative abundance of a protein could be compared based on its APEX value.

2.6. Real time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was separately extracted from the MSG-A, -M, and -P using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China) was used for the reverse transcription experiment. RT-qPCR was carried out with a LightCycler® 480 (Roche Diagnostics, Switzerland) in a 20-μL reaction volume containing 50 ng of cDNA, 10 μL of 2× SYBR Premix Ex Taq™ (TaKaRa, Dalian, China), and 4 μM each of the forward and reverse primers (Supplementary Table 1). The thermocycler program began at 95 °C for 30 s for DNA denaturation, followed by 40 cycles of amplification with 95 °C for 5 s, 60 °C for 20 s, and 72 °C 15 s. The relative gene expression level was calculated based on the delta Ct value using the $2^{-\Delta CT}$ method [28]. GAPDH (accession no. NM_001043921) was used as a reference gene. The statistical analysis of gene expression was performed by using SPSS software (Version 18). Multiple comparison for MSG-A, -M, and -P was analyzed with Duncan's test. Comparison between MSG and PSG was done with Student's t test. P values < 0.05 were taken to be statistically significant.

2.7. Bioinformatic analysis

Gene Ontology (GO) terms for the identified proteins were retrieved by searching against the latest InterPro member databases using InterProScan software. GO annotations of the proteomes were plotted by subjecting the retrieved GO terms in native format to the Web Gene Ontology Annotation Plot (WEGO) website (http://wego.genomics.org.cn/cgi-bin/wego/index.pl). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed with the Molecule Annotation System (MAS 3.0, http://bioinfo.capitalbio.com/mas3/).

3. Results

3.1. Proteome profiling of the different MSG sections

For better understanding the molecular details of MSG function, the whole proteomes of MSG subsections were analyzed by shotgun LC–MS/MS (Fig. 1A). There were no significant differences in the expression patterns among the three subsections samples separated by SDS-PAGE (Fig. 1B). Stringent filtering and manual checking were done after protein identification. We totally identified 8078, 7125, and 9600 peptides with a minimum probability of 0.7 from the MSG-A, -M, and -P, respectively (Supplementary Tables 2–4). Assembled with