Analysis of proteome dynamics inside the silk gland lumen of *Bombyx mori*

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The silk gland is the only organ where silk proteins are synthesized and secreted in the silkworm, *Bombyx mori*. Silk proteins are stored in the lumen of the silk gland for around eight days during the fifth instar. Determining their dynamic changes is helpful for clarifying the secretion mechanism of silk proteins. Here, we identified the proteome in the silk gland lumen using liquid chromatography–tandem mass spectrometry, and demonstrated its changes during two key stages. From day 5 of the fifth instar to day 1 of wandering, the abundances of fibroins, sericins, seroins, and proteins of unknown functions increased significantly in different compartments of the silk gland lumen. As a result, these accumulated proteins constituted the major cocoon components. In contrast, the abundances of enzymes and extracellular matrix proteins decreased in the silk gland lumen, suggesting that they were not the structural constituents of silk. Twenty-five enzymes may be involved in the regulation of hormone metabolism for proper silk gland function. In addition, the metabolism of other non-proteinous components such as chitin and pigment were also discussed in this study.
Results

Extraction and identification of proteins in the silk gland lumen. The silk glands were dissected out from silkworms at two different developmental stages: day 5 of the fifth instar (V-5) and day 1 of wandering (W-1). Each silk gland was cut into five compartments according to morphology (Fig. 1A), including the anterior silk gland (ASG), the anterior part of the middle silk gland (A-MSG), the middle part of the middle silk gland (M-MSG), the posterior part of the middle silk gland (P-MSG), and the posterior silk gland (PSG) (Fig. 1A). Each compartment was then divided into two parts, the glandular cells and the luminal contents (Fig. 1A,B). The luminal contents from different compartments showed a wide variety of diameters. In particular, the M-MSG had the maximum diameter of 2 mm, and the ASG had the minimum diameter of less than 0.1 mm (Fig. 1B). The luminal contents of the M-MSG was surrounded by a glandular cell “sheath” of a similar size, but the luminal contents of the ASG were much thinner than its glandular cell “sheath” (Fig. 1B), because the ASG contains a thick duct wall made of chitin which narrows the lumen.

Results of SDS–PAGE revealed several similar, intensely-stained protein bands in the lumen of M-MSG, P-MSG, and PSG (Fig. 1C), which mainly included the 350 kDa fibroin heavy chain, the 400 kDa or 150 kDa sericin, the 26 kDa fibroin light chain, and the 27 kDa or 30 kDa fibroin p25 protein. Fibroins and sericins were at low abundance in the lumen of ASG and A-MSG on day 5 of the fifth instar, but their level obviously increased on day 1 of wandering (Fig. 1C). A dozen protein bands seems similar between the ASG and A-MSG on day 5 of the fifth instar (Fig. 1C).

LC–MS/MS was used to determine the proteome in the lumen of five silk gland compartments during two developmental stages. With the combined analysis of triplicate samples, we identified 10,747 tryptic peptides, which assembled to 1,271 proteins (Supplementary Dataset 1 and Dataset 2). The average number of peptides per protein was 8.5, leading to an average sequence coverage of 27.6% (Supplementary Dataset 2). Most proteins (94.2%) were identified by two or more unique peptides. On day 5 of the fifth instar, we identified 548, 446, 724, 797, and 645 proteins in the ASG, A-MSG, M-MSG, P-MSG, and PSG, respectively (Supplementary Figure S1). On day 1 of wandering, we identified 502, 839, 402, 387, and 716 proteins in the ASG, A-MSG, M-MSG, P-MSG, and PSG, respectively (Supplementary Figure S1). In contrast to day 5 of the fifth instar, substantial numbers of proteins increased in the A-MSG on day 1 of wandering, but decreased in the M-MSG and P-MSG on day 1 of wandering.

Annotation of proteins in the silk gland lumen. The Blast2GO analysis tool (version 2.6.6) was used to subject the luminal proteins to GO annotation (Supplementary Dataset 2), and revealed that the identified proteins were involved in metabolic process, transcription and translation, ion transport, protein transport, stress response, signal transduction, extracellular matrix, regulation of proteolysis and cytoskeleton organization. The Phobius server was used to predict the transmembrane regions and signal peptides, and identified 868 intracellular proteins, 262 extracellular proteins, and 141 transmembrane proteins (Supplementary Dataset 2).
identified intracellular proteins in the lumen of the silk gland might represent some leakage from cells of the silk gland. Although only 262 proteins were predicted to be extracellular proteins, they accounted for 87.0\(^\text{-}99.0\%\) of the total protein abundance when being investigated with the intensity-based absolute quantification (iBAQ) intensity (Fig. 2A,B). Furthermore, we found that 295 proteins in the silk gland lumen were also detected in the scaffold silk and cocoon silk by previous studies\(^7\),\(^3\), making up 93.4\(^\text{-}99.5\%\) of all protein molecules in the silk gland lumen (Fig. 2A,B).

Protein quantification results based on the functional classification indicated that proteins in the silk gland lumen mainly consist of fibroins, sericins, seroins, extracellular matrix proteins, protease inhibitors, enzymes, and proteins of unknown function, of which fibroins were the most abundant components (Fig. 2C and Supplementary Dataset 2). Proteins from each functional category increased or decreased in different compartments of the silkworm gland lumen from day 5 of the fifth instar to day 1 of wandering (Fig. 2C).

Quantitative comparison of proteins in the silk gland lumen between two key stages. From day 5 of the fifth instar to day 1 of wandering, twelve proteins showed greatest increment based on intensities (Fig. 3 and Supplementary Table S1), including three fibroins, two sericins, three proteins of unknown functions, three protease inhibitors and one seroin. From day 5 of the fifth instar to day 1 of wandering, these twelve proteins increased in different compartments of the silk gland lumen (Fig. 3 and Supplementary Table S1): three fibroins (fibroin H, L and p25) jointly increased in the ASG, two sericins (sericin 1 and 3) increased in the ASG and A-MSG, three protease inhibitors (serine protease inhibitor BmSPI39 and BmSPI51, and carboxypeptidase inhibitor) mainly increased in the A-MSG, osifirs-9-like protein increased in ASG, A-MSG, and M-MSG, glycine cell wall structural protein 1.0-like protein and fibroin p25 like protein increased in the M-MSG and P-MSG, whereas seroin 1 increased in all the silk gland. As a result, these twelve proteins in the silk gland lumen constituted the twelve most abundant cocoon proteins, accounting 94.0\(^\text{-}96.8\%\) of the total protein abundance\(^3\). From day 5 of the fifth instar to day 1 of wandering, eleven luminal proteins showed greatest reduction (Fig. 4 and Supplementary Table S1), which were abundant in the ASG and A-MSG lumen on day 5 of the fifth instar, but nearly disappeared on day 1 of wandering. All the eleven proteins significantly decreased \((P < 0.05)\) in the ASG.
Hormone metabolism enzymes were identified in the silk gland lumen. Hormones play important roles in the regulation of the development and function of silk gland. Fortunately, we identified twenty-five enzymes in the silk gland lumen that may be involved in the hormone metabolism (Supplementary Table S2). Among them, eleven juvenile hormone esterase (JHE) and JHE-like proteins, three juvenile hormone epoxide hydrolases (JHEH) and JHEH-like proteins may be involved in the juvenile hormone metabolism, while eight ecdysone oxidase (EO) and EO-like proteins, one 3-dehydroecdysone 3 alpha-reductase (3DE-3α-R), and two 3-dehydroecdysone 3 beta-reductase (3DE-3β-R) like proteins may play roles in the molting hormone metabolism.

The results of semi-quantitative RT-PCR confirmed that fifteen hormone metabolism enzymes were expressed in the silk gland (Fig. 5A). It was noteworthy that day 5 of the fifth instar and day 1 of wandering were really two key stages, because that most of hormone metabolism enzymes had obvious expression change during the two stages (Fig. 5A). Their change in mRNA level was almost the same as the variation in protein level (Fig. 5B).

Furthermore, protein quantification results suggested that most of hormone metabolism enzymes were secreted into the lumen of ASG and MSG (Fig. 5B), and twelve of which had been detected in the silk (Fig. 5C). According to the insect hormone biosynthesis pathways in the KEGG database and insect pathway database, we speculated that juvenile hormone (JH) may be metabolized to inactive JH acid, JH diol, and JH acid diol by JHE and JHEH, whereas ecdysone may be metabolized to 3-dehydroecdysone and 3-epiecdysone by EO and 3DE-3α-R in the silk gland lumen (Fig. 5D). It is a known fact that hormones control the development of silk gland and synthesis of silk, whereas the hormone metabolism enzymes play important roles in the regulation of the hormonal titer.

Discussion
Silk proteins are synthesized and secreted by silk gland cells, and stored in the lumen of the silk gland for around eight days during the fifth instar. After that, silk proteins were spun out from the anterior silk gland to form silk.
Fibroin heavy chain associated with the light chain fibroin and the P25 glycoprotein are produced by the PSG cells and constitute the core silk fiber. Besides fibroins, abundant seroin 1 were also identified in the PSG lumen, indicating that seroin 1 may have important functions in the PSG. Although seroin 1 was reported to play antimicrobial roles\(^4^0\), it may have other functions, possibly similar to that of p25 protein\(^4^1\), playing a role to assemble fibroin heavy chains and light chains into an elementary silk unit\(^1\). When the fibroins flow into the MSG lumen, several layers of sericins are subsequently added to the fibroin core. Sericin P (150 kDa), sericin M (400 kDa) and sericin A (250 kDa) was identified in the P-, M- and A-MSG sections, respectively\(^2^8\). Thus, they correspond to the internal layer, middle layer and external layer of sericin, respectively\(^6\). Sericin M and sericin P were identified as products of the sericin 1 gene and sericin A was found as the product of sericin 3 gene\(^4^2\). Sericin 2, unlike the sericin 1 and 3, decreases on day 1 of wandering, which may be pushed into the spinneret by pressure from the accumulated proteins in the lumen. This speculation is consistent with its location and function: sericin 2 was the major coating proteins of non-cocoon silk, which was detected in the scaffold silk, the silk spun before cocoon construction\(^5\),\(^7\),\(^4^3\).

Three proteins with unknown functions were identified as the major cocoon proteins, which have similar sequence characteristic with fibroins but distinct spatial distribution. Glycine-rich cell wall structural protein 1.0-like is rich in glycine (36.5%) and alanine (12.5%) residues, liking the fibroin heavy chain, but has small protein size as 18.5 kDa. Osiris-9 like protein is rich in leucine (15.9%) and alanine (10.5%). Fibroin p25-like protein showed 53% identities with the fibroin p25. From day 5 of the fifth instar to day 1 of wandering, glycine-rich cell wall structural protein 1.0-like increased in the M-MSG lumen, fibroin p25-like protein increased in the M-MSG and P-MSG lumen, whereas osiris-9 like protein increased in the ASG, A-MSG and M-MSG lumen (Fig. 3). All the three proteins were identified as the major cocoon components\(^3^2\), but have uncharacterized functions.

Two protease inhibitors BmSPI51 and BmSPI39 increased before spinning, and mainly distribute in the A-MSG, corresponding to the external sericin layer. Previous studies found that BmSPI51 had high inhibitory
activity against animal trypsin, and its homolog in *Galleria mellonella* could inhibit both the trypsin and fungal proteases (subtilisin and proteinase K). BmSPI39 could significantly inhibit fungal proteases and spore germination of *Beauveria bassiana*. Moreover, a recent study proved the protease inhibitors in the cocoon could inhibit trypsin and proteinase K but not chymotrypsin and elastase, implying that they may be important in preventing the cocoon destruction by trypsin and fungal proteases. More protease inhibitors in the external sericin layer allow the cocoon to provide better protection from the predatory invasion.

Inside the ASG lumen, the chitin and chitin-binding cuticular proteins form an extracellular matrix layer to protect the gland cells from being damaged by high mechanical shear of spinning, whereas shear force is important to induce self-assembly of silk proteins into fibrils. The decrease of cuticular proteins on day 1 of wandering may reflect that incompact cuticular proteins have been assembled into a tough protective layer by cross-linking and binding to chitin. Oxidases are responsible for the cross-linking of cuticular proteins, while protease and chitinase may be involved in the degradation of extracellular matrix layer. One recent study found that a serpin-type protease inhibitor BmSPI16 can regulate the activities of cysteine proteases in the silk gland. Therefore, we speculated that some protease inhibitors, such as BmSPI16 and BmSPI38, may play roles in protecting stored silk proteins inside the lumen from unexpected degradation. From day 5 of the fifth instar to day 1 of wandering, some protease inhibitors such as BmSPI16 and BmSPI38 decreased, little of which could be detected in the cocoon, whereas some protease inhibitors such as BmSPI51 and BmSPI39 increased and became the major cocoon components. It may be due to that they have divided roles to protect the silk proteins in the silk gland or in the cocoon.

Besides chitin, other non-proteinous components such as pigments are also actively secreted in the silk gland, and accumulate in the sericin layers of cocoon. The cocoon pigments vary depending on the *B. mori* strain. Some silkworm strains produce yellow-green cocoon shells containing flavonoid pigments.

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**Figure 5. Hormone metabolism enzymes in the silk gland lumen.** (A) Expression patterns of seven juvenile hormone metabolism enzymes and eight molting hormone metabolism enzymes. Semi-quantitative RT-PCR was performed using gene-specific oligonucleotides (Supplementary Table S3). The silkworm housekeeping gene ribosomal protein L3 (*BmRpl3*) was used as internal control (Liu et al. 2010). (B) The relative abundances of hormone metabolism enzymes in the silk gland lumen were compared according to the normalized iBAQ intensity (Supplementary Table S2). (C) Hormone metabolism enzymes detected in the scaffold silk and cocoon silk (Dong et al. 2013). (D) Hormone metabolism pathways in the silk gland lumen. This figure presents both the juvenile hormone metabolism pathway and molting hormone metabolism pathway according to the insect hormone biosynthesis pathways in the KEGG database and insect pathway database (iPathDB).
DaZao strain used in this study\textsuperscript{51}. Flavonoid pigments could be observed in the middle silk gland of Dazao strain as in Fig. 1B. The flavonoids in the mulberry leaves were absorbed by the silkworm midgut, and then glucosylated in the midgut and silk gland\textsuperscript{52}. In this study, four UDP-glucosyltransferase were identified in the silk gland, which could transfer glucose to the hydroxyl groups of flavonoids\textsuperscript{52}. The mechanisms for transport of flavonoids are not well understood in any animal system, but the glucosylation at the 5-O position was speculated as the key step to allow or facilitate the efficient uptake and transport of flavonoids from midgut to silk gland\textsuperscript{53}. The glucosylated flavonoids may be used to increase the anti-oxidative state of the tissues and increase the UV-shielding activity of cocoons\textsuperscript{52,53}.

Twenty-five enzymes identified in this study may be involved in the hormone metabolism. Both the JHE and JHEH are responsible for the degradation of JH (Fig. 5D). JHE and JHE-like genes continuously expressed in the silk gland from day 0 of the fifth instar to day 5 of the fifth instar (Fig. 5A), which were considered to be controlled by hemolymph JH titer\textsuperscript{38}. The expression of JHEH and JHEH-like genes are probably regulated in similar manners, because their expression profiles are similar to that of JHE and JHE-like (Fig. 5A). On day 1 of wandering (day 7–8 of the fifth instar), some JH metabolic enzymes were down-regulated at both the mRNA and protein level (Fig. 5A), which may be due to the rise of ecdysteroids\textsuperscript{38}. The secretary JHE in the hemolymph were reported in previous studies\textsuperscript{54–56}, whereas the secretary JHE and JHEH in the silk gland lumen were found for the first time. We can not rule out the possibility that the detection of these enzymes in the silk gland lumen might due to their leakage from silk gland cells\textsuperscript{5}. However, six of the JH metabolic enzymes were predicted to have signal peptides, indicated that they may have roles in the extracellular matrix to degrade JH around cells\textsuperscript{38}.

EO and 3DE-3β-R play roles to degrade the molting hormone (ecdysteroids), whereas 3DE-3β-R is involved in the biosynthesis of ecdysteroids (Fig. 5D). The ecdysteroids maintain low level during the feeding stage, which is necessary for proper ecdysteroids on day 1 of wandering, ecdysteroids metabolic enzymes showed corresponding fluctuation, decrease or increase, in the silk gland (Fig. 5A). Two EOs were identified in the silkworm\textsuperscript{59,58}, both of which belong to the family of glucose–methylol–choline oxidoreductase. Sun et al. considered that BmEO/BmGMC2 (BGIBMGA000158) may have not EO activity, because it only has 1 of 5 conserved ecdysone-binding residues\textsuperscript{58}. Therefore, the activities of these putative ecdysteroids metabolic enzymes are still to be validated in future.

Methods

Sample collection and electrophoresis. The silkworm strain DaZao was reared on mulberry leaves at 25°C. The silk glands were dissected out at 4°C in 0.75% (w/v) NaCl on day 5 of the fifth instar and day 1 of wandering, before spinning. They were frozen in liquid nitrogen for 2 min and then immersed in 60% pre-cool ethanol for 2 min. The silk gland was divided into five compartments: ASG, A-MSG, M-MSG, P-MSG, and PSG (Fig. 1A). The solid luminal contents were then dragged out of the exterior silk gland cells. The luminal contents from twenty individuals were collected as one sample, and were dissolved in 9 M LiSCN with vortexing for 2 h. The solubilised proteins were recovered by centrifugation (12,000 g, 10 min, 4°C). The protein concentrations in sample collection and electrophoresis.

Protein digestion and LC-MS/MS. The luminal proteins (60 μg) were digested according to previously reported methods\textsuperscript{7,32,60,61}. The resulting tryptic peptides were recovered by centrifugation in the ultrafiltration tube, lyophilized, and resuspended in 35 μL of 0.1% formic acid. The tryptic peptides (6.5 μL) were separated on the Thermo Fisher Scientific EASY-nLC 1000 system using a Thermo Fisher Scientific EASY-Spray column, with a 120 min gradient consisting of 2 min at 3%–8% buffer B (100% acetonitrile, 0.1% formic acid), 80 min at 8%–20% buffer B, 10 min at 20%–30% buffer B, 5 min at 30%–70% buffer B, 3 min at 70%–90% buffer B, and 20 min at 90% buffer B. The separated peptides were analysed with a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer operating in data-dependent mode. The instrument parameters were as follow: the resolution was 70,000 for full MS scan and 17,500 for MS² scan; the automatic gain control target was 3E6 for full scan and 1E6 for MS²; the maximum ion injection time was 20 ms for full MS scan and 60 ms for MS² scan. Three biological replicates were used for the LC-MS/MS analyses.

Protein identification, quantification and annotation. The resulting raw MS data were analysed with the MaxQuant software (version 1.3.0.1)\textsuperscript{62}. The MaxQuant searches were executed against an integrated silkworm proteome database containing 35,379 protein sequences from NCBI and silkDB. Peptide searches were performed with the Andromeda search algorithms\textsuperscript{63}. The search parameters were set as reported previously\textsuperscript{7,32,60,61}. A minimum of one unique peptide was required for the identified protein. All common contaminants and reverse hits were removed. The identified peptides and proteins are listed in Supplementary Dataset 1 and Dataset 2, respectively.

The iBAQ algorithm in MaxQuant was used to determine the protein abundances\textsuperscript{64}. We assumed that the total intensity of each sample was same (its intensity was set as 100%), and then normalized the relative intensity of each protein. The estimates of protein intensity were presented in Supplementary Dataset 2. Heat map of protein abundance was generated using the HemI (Heatmap Illustrator, version 1.0.3.3)\textsuperscript{65}. Two-tailed t-test was used to determine the differential expression between day 5 of the fifth instar and day 1 of wandering.

To annotate the molecular functions of proteins, we used the Blast2GO software (version 2.6.6)\textsuperscript{30}, an all-in-one program for performing Blast searches, Gene ontology (GO) annotation, enzyme code (EC) annotation, signal peptides prediction, transmembrane domain prediction and KEGG pathway construction. The default settings of Blast2GO were used in every step.
Temporal expression analysis of hormone metabolism enzymes in the silk gland. The silk glands were collected at five different developmental stages: day 0 of the fifth instar, day 1 of the fifth instar, day 3 of the fifth instar, day 5 of the fifth instar, day 1 of wandering. Total RNA was isolated using TRIzol reagent (Invitrogen, USA). Contaminating genomic DNA was digested using RNase-free DNAse I (Promega) for 30 min at 37 °C. Total RNA (10 μg) was reverse-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen, USA) at 42 °C. All cDNA samples were normalized using B. mori housekeeping gene ribosomal protein L3 (BmRPL3) as an internal control (forward primer: 5’-TCG TCA TCG TGG TAA GGT CAA-3’; reverse primer: 5’-TTT GTA TCG TTT GCC CTG GGT-3’). The primers for semi-quantitative RT-PCR detection are listed in Supplementary Table S3. PCR amplification was performed in a total reaction volume of 25 μL using the following program: initial incubation at 94 °C for 4 min, followed by 28 cycles of 40 s at 94 °C, 40 s of annealing (52–58 °C), 30–50 s of extension (72 °C), and a final extension at 72 °C for 10 min. Aliquots of 5 μL of the PCR products were separated on 1.3% agarose gels and stained with EB.

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