Ectopic expression of sericin enables efficient production of ancient silk with structural changes in silkworm

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Article

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Abstract

Bombyx mori silk is a super-long natural protein fiber with a unique structure and excellent performance. Innovative silk structures with high performance are in great demand, thus resulting in an industrial bottleneck. Herein, a transgenic method was used in which the outer layer sericin SER3 in silk is secreted into the inner fibroin layer, thus generating a new structural fiber with non-fibrous sericin microsomes dispersed in fibroin fibrils. The water-soluble SER3 protein secreted by the posterior silk gland causes P25's detachment from the fibroin unit of the Fib-H/Fib-L/P25 polymer and accumulation on the fibroin surface. Moreover, the water solubility and stability of the fibroin-colloid in the silk glandular cavity are increased, thus significantly improving the β -sheet content of fibroin, as well as the mechanical properties, moisture absorption and moisture liberation of the silk fiber. Our silkworm mutant system circumvents the problems of low vitality and abnormal silk gland development, and enables higher production efficiency of cocoon silk than that of the wild type. We describe a silk gland transgenic target protein selection strategy to alter the ancient silk fiber structure and to innovate the properties of silk protein materials. This study thus provides an efficient, green method to produce new silk fibers.

Introduction

The beautiful silk fibers produced by the silkworm (*Bombyx mori*) have excellent performance and are an easily available renewable protein material. The toughness of silk fiber and its unusual combination of high strength and expansibility have not been surpassed by synthetic materials to date^{1,2}. The silk gland (SG) in silkworm larvae is the most efficient insect organ for protein synthesis and exocrine secretion; it can synthesize 20–35% of its own weight in protein in approximately 1 week in 5th instar larvae³. The concentration of aqueous silk protein solution in the SG cavity is as high as 30%. This fiber processing unit, which maintains a metastable state of ultra-high-level protein, is difficult to recapitulate through modern textile engineering technology^{4,5}. Simulating the biological template of SG has emerged as a new research direction for developing high-performance, multifunctional protein fiber materials through green chemical processing. The multifunctional materials processed from silk, such as hydrogels, fibers, sponges, films and other forms, has been used in many applications, such as medical materials, electronic information and fine chemicals, thus demonstrating broad application potential^{1,2,6}.

Although many important insights in the synthesis and self-assembly of silk proteins have been obtained in the past 10 years^{7–13}, understanding remains lacking regarding the mechanism of the metastability of ultra-high concentration aqueous solutions of Fib-H/Fib-L/P25 polymers in SGs. Many advances and engineering applications have extended the functions of silk fibers, including silk processing by chemical or physical methods, and obtaining biomaterials for many purposes by modulating the self-assembly properties of silk fibroin^{14–19}. However, these achievements have been based on the reprocessing and transformation of the ancient silk structure. The future advancement of related technologies and achievements will depend on breakthroughs in altering the ancient silk structure.

The germplasm resources for mutant genes associated with silkworm cocoon silk purification have a long history of thousands of years, and the cultivation of hybrid varieties has also been performed for hundreds of years. These efforts have greatly contributed to optimizing the fiber characteristics of silk. However, owing to a bottleneck in the homogenization of silkworm varieties, the silk fibers produced by thousands of silkworm varieties worldwide have nearly the same composition, structure and characteristics^{20,21}. Therefore, innovative reprogramming of the genomes of SG cells to alter the structure and characteristics of the silk fiber is highly desirable^{21,22}. An attractive method is to directly integrating special functional fiber protein genes into the silkworm genome has been described to aid in achieving high-efficiency expression in SGs, such as the expression of a high-strength spider silk protein gene in silkworm SGs to obtain silk fibers with improved mechanical properties^{23–26}, and the expression of fusions of optical functional protein and silk protein to obtain photoelectric silk or fluorescent silk^{27,28}. However, the efforts to express and secrete exogenous proteins in the SGs of silkworms through transgenic technology to date have generally resulted in low efficiency silk protein synthesis and secretion, at expression levels far below those of normal silk proteins (Supplementary, Table 1). The performance of new structural silk produced by transgenic silkworms is far inferior to that of fibers produced by donor animals. Moreover, the problems of SG deformity and declining silk production efficiency are common^{29,30}. The strategy of directly introducing functional silk protein genes from other organisms or similar artificially designed genes into the silkworm genome to produce new silkworm silk in the SG has been problematic. In this study, a new strategy was developed. Through expression of the silkworm's own sericin protein in the PSG, the fibril structure and function of the ancient silk fiber were greatly altered, and a new type of silk fiber was obtained. This method may help address the bottleneck problems of the low survival rate and low silk yield of genetically transgenic silkworms.

Results

A genetically modified silkworm mutation system to alter silk fiber structure. The structure and formation of silkworm silk fiber is shown in Fig. 1a. The water-soluble Sericin (SER3) protein secreted by the anterior part of the middle silk gland (MSG) and wrapped in the outermost layer of the silk is not present in the fibroin fibrils. The technical strategy of this study involved specifically expressing SER3 recombinant protein in silkworm PSG cells to achieve secretion into the PSG lumen and the incorporation of water-soluble SER3 into the silk fibroin colloidal solution, thus altering the metastable state of silk fibroin protein polymers and further affecting the structure of silk fibers after self-assembly (Fig. 1b, c). Using red fluorescence in the eyes and green fluorescence in silk fibers as markers, after six consecutive generations of screening, we obtained the SER (SER3/SER3) mutant system (Supplementary Fig. 1b-d). In the PSG cells of the SER larvae, the mRNA and SER3 protein expressed by the *Ser3* gene were detected, and the results were consistent with those from screening of the RFP/EGFP reporter genes (Supplementary Fig. 1e, f). Tail-PCR detection revealed that a single copy of the piggyBac transposon was inserted into the *Bombyx mori* genome at the non-functional gene sequence at Chr.23 (scaf12: 4699379...4699384) (Supplementary Fig. 1g). Many sericin microsomes (SM) were present in the fibroin of silk fibers produced by SER silkworms, and vacuoles were observed in the SM (Fig. 1b). Continued

investigation for 12 generations revealed that the mutant silkworms showed stable growth and development, and the production efficiency of cocoon silk was significantly higher than that of the wild type (WT). We observed no SG shortening, deformities or decreased individual survival rates, which are common problems in SG transgenic silkworms (Supplementary Fig. 2). From the perspective of sericulture, our findings demonstrate that the transgenic silkworm SGs have superior production performance.

Mutant silkworms show silk fiber structure rearrangement and performance improvement. The silk fibers produced by the mutant silkworms exhibited green fluorescence from an EGFP fusion with SER3. As observed from a cross-section of the silk fiber, the fluorescence distribution was uneven, and strong fluorescence appeared on the surface, in both the inner silk fibroin area and the outer sericin layer (Fig. 2a). The longitudinal section of the silk fiber was observed by transmission electron microscopy (TEM). Large amounts of SER3-EGFP microsomes (SM) in the SER group were dispersed in the silk fibroin area. The SM shape was rain-thread-like or fusiform, and the shape was altered in the same direction as the movement of silk protein colloid under squeezing during the spinning process in mature larvae. Notably, in SM, vacuoles of low-density silk protein aqueous solutions of different sizes and shapes were observed (Fig. 2b). The cross-sectional TEM images further confirmed the presence of SM and vacuoles in the mutant silk fibers (Supplementary Fig. 3). The percentage of sericin in cocoon silk in the SER group was 7.39% higher than that in the WT group (Fig. 2c), an increase in 21.8%. Our results indicated that the PSG of the mutant silkworm synthesized the SER3 protein very efficiently and successfully secreted it into the silk fiber.

Using immunofluorescence, we observed that the P25 protein in the WT silk fiber was evenly distributed in the silk fibril area, whereas in the silk fibers of SER, almost all P25 had transferred to the outside of the silk fibrils and was unevenly distributed between the silk fibroin layer and the sericin layer, with different micro-body sizes (Fig. 2d). Our findings suggested that P25 in SER silk fibers was separate from the silk protein comprising Fib-H/Fib-L/P25 polymers, thus indicating that the ordered fibril structure in the silk protein was greatly altered by the influence of the SER3 protein synthesized in the PSG.

The protein secondary structure of silk fibers was analyzed by FTIR. After deconvolution of the amide I band (Fig. 2e) in the silk fibers of mutant SER, we observed that the content of β -sheets increased significantly, whereas the content of random coil and α -helices decreased significantly (Fig. 2f). These results suggest that the β -sheet level positively correlated with the rigid composition of silk fibers, and the random coils and α -helices, which are positively associated with mechanical properties such as silk fiber ductility, might also have been altered accordingly.

The amino acid composition of silk fiber was analyzed. We observed no difference in amino acid composition between the cocoon silk of SER and WT. However, the silk fiber containing sericin protein showed changes in the relative content of a variety of amino acids, such as increased relative content of serine and aspartic acid and decreased relative content of glycine, alanine and tyrosine. In the silk fiber (fibroin) for textile raw materials after removal of the outer sericin, the content of alanine increased by

only 1.7% (29.9% in WT versus 30.41% in SER), and the relative content of other amino acids scarcely changed (Supplementary Table 2), because the amino acid composition of Fib-H/Fib-L/P25 polymers of silk fibroin is the same as that of SER3, and the relative content is also similar (Supplementary Table 3).

The mechanical properties of fibroin fibers after removal of the outer sericin were analyzed. The stress and strain curve indicated that the tensile initial modulus of the SER group increased significantly, from 73.48 MPa to 110.93 MPa, a value 1.51 times higher than that in the WT group (Fig. 3a). The maximum stress level (Fig. 3b) and Young's modulus (Fig. 3d) in the SER group were also significantly higher than those in the WT group. Only the maximum elastic modulus had no statistically significant change (Fig. 3c). The fibroin fibers produced by mutant silkworms thus had better mechanical properties and better shaping effects for textile materials.

The moisture absorption and desorption performance of fibroin showed significant improvements in the SER group. The moisture absorption curve (Fig. 3e) and dehumidification curve (Fig. 3g) of silk fiber in the SER group were highly similar to those in the WT group. The moisture absorption and regained dehumidification were 22.0% and 8.0% higher, respectively, than those in the WT group. The moisture absorption rate and moisture liberation rate in 0–1 min were 142.5–139.4% (Fig. 3f) and 165.5–164.1% (Fig. 3h) those of the WT group, respectively.

SEM characterization indicated that the adhesion between silk fibers in the SER cocoon silk layer was closer, and the pores were smaller than those in the WT (Fig. 3i). After removal of sericin with the alkali method, the surfaces of fibroin fibers in the SER group were smoother, and less fibril damage was observed than that in the WT (Fig. 3j). The results showed that the silk fibers in the SER group were more alkali resistant than those in the WT group.

Biocompatibility testing indicated that fibroin fibers showed no adverse effects on the proliferation and growth of mammalian cells. Fibroin and L929 cells were co-cultured for 48 hours. The cell growth state (Fig. 3k) and the proportion of dead cells (Fig. 3l), as determined by Live-Dead staining, indicated that the fibroin fibers of SER were significantly better than the medical non-absorbable suture (NASS), and no statistical difference was observed relative to WT fibroin and the negative control (null). The MTT test results also indicated that the number of L929 cells in the SER group was significantly higher than that in the NASS group (Fig. 3m). The content of the pro-inflammatory factor nitric oxide in the culture medium was found to be significantly lower in the SER group than the NASS group (Fig. 3n). SER silk fibroin had good biocompatibility, as compared with classical silk fibroin, although sericin SER3 protein had been introduced.

Enhanced water solubility and stability of the silk fibroin colloid in the mutant PSG. Frozen sections were used to observe the SGs with the most vigorous stage of silk protein synthesis in the 5th instar 3rd day larvae, and the distribution of SER3 was assessed via the EGFP fusion protein (Fig. 4a). In the PSG lumen of the mutant larvae, we observed fluorescent particles of different sizes and shapes, with diameters of several micrometers (1–5 μm), scattered in a liquid comprising a grid of bubbles. The water-soluble EGFP-SER3 fusion protein distributed in the silk protein aqueous solution also entered the fibroin mass in

an aggregated state. In the MSG lumen, the green fluorescence was distributed in both the fibroin and the sericin, but the fluorescence was stronger in the boundary area between the silk fibroin layer and the sericin layer. Notably, the fluorescent particles increased to tens of micrometers (10–50 μm) in diameter, and the bubble grid-like characteristics of the liquid distribution in the PSG lumen disappeared. The fluorescence distribution pattern in the ASG lumen indicated that the fluorescence in the outer layer of sericin was weak, and the distribution of fluorescent particles in the inner layer of silk fibroin tended to be uniform, but the sizes remained different, and the diameter decreased to 1–5 μm . On the microvilli in the MSG lumen and ASG, droplet-like green fluorescence was observed with a higher intensity than that in the sericin of the middle layer. With the movement of silk protein from the PSG to the ASG via the MSG, the aqueous solution of EGFP-SER3 fusion protein was incorporated into the forming fibroin mass, and the colloidal aggregation state of sericin (SER3) significantly changed, appearing in size and shape different fluid sericin microsomes. The structure and morphology of the fluid SER3 protein microsomes are shown in Fig. 2b and Supplementary Fig. 3.

TEM was used to observe the substructure of the SG cells and the secretion of silk protein in the 5th instar larvae (Fig. 4b). The organelles of the mutant PSG cells were normal, and appeared to be identical to those in the WT, with abundant rough endoplasmic reticulum, Golgi apparatus, mitochondria and other subcellular structures, thus indicating normal protein synthesis. The significant difference was that in the mutant PSG cells, the storage silk protein layer of was thinner than that in the WT cells, and the amount of fibroin secreted into the glandular cavity was much greater. Few spherical aggregates of fibroin mass were observed in the lumen, and the silk protein colloids were more evenly distributed. Thus, the SER3 protein expression in the PSG improved the water solubility of the silk fibroin colloid.

The gene transcription levels of *EGFP*, *Ser3*, and the silk fibroin components *Fib-H*, *Fib-L* and *P25* in different parts of the SG cells were measured (Fig. 4c-4e). The PSG cells of the 5th instar larvae of the mutant efficiently expressed the *Ser3* gene, which is specifically expressed in the WT silkworm MSG (MA and MM). In PP cells, the transcription level of the *Ser3* gene reached that in MM cells. Notably, the mRNA of the *Ser3* gene was detected in MP cells of SER 5th instar larvae, although the transcription level was only 1–5% that of PSG cells (Fig. 4c-4e), similarly to the fibroin genes expressed in the MP cells of both WT and SER 5th instars (Fig. 4c). Our results indicated that the *Fib-H* promoter used by the transgenic mutant expressed the SER3 and EGFP genes in MP cells and accounted for the strong green fluorescence observed in the outer sericin in the MSG lumen in Fig. 4a.

Discussion

The PSG expresses water-soluble sericin enabling the sustainable production of novel silk fibers with controllable changes in their structure and properties. Herein, the *Ser3* gene specifically expressed by the silkworm MSG was expressed in the PSG. The amino acid composition of the SER3 protein was the same as that of silk fibroin, and the relative amino acid content was also highly similar, thus avoiding imbalances in amino acid supply in the mutant PSG cells. The ratio of the number of cysteine molecules in the amino acid residues of the SER3 protein (0.50%) was intermediate between that of *Fib-H* (0.10%)

and Fib-L (1.10%), which can form disulfide bonds and combine with Fib-H and other silk fibroin in the PSG. The SER3 protein expressed in the PSGs was present in the long cocoon silk fibers composed of silk fibroin protein polymers. SER3 is a water-soluble protein wrapped in the outer layer of silkworm silk fibers. It does not exist in the fibroin layer, does not contact the fibrils and is completely dissolved by hot water during the silk reeling process. In the silk fiber produced by the mutant (Fig. 2), we observed SER3 protein microsomes dispersed among the silk fibrils in many different droplet sizes, thus indicating that the fibrils were not broken but were incorporated into other silk proteins. In the PSG of the SER silkworm larvae, less fibroin mass was retained in the gland cells, and we observed few spheroid aggregations of fibroin mass in the lumen and silk fibroin colloids, which were more evenly distributed (Fig. 4). Our findings demonstrated that the hydrophilic SER3 protein synthesized and secreted by PSG improves the water solubility and stability of the silk fibroin colloid in the SG cavity, and further affects the polymerization and fibrogenesis of silk fibroin.

Studies have shown that the silk fibroin protein synthesized by silkworm PSG is present as fibroin units of Fib-H/Fib-L/P25 (molecular ratio 6:6:1) in silk fibers⁹. Among these proteins, P25 can form intermolecular interactions with Fib-H/Fib-L⁷ and is evenly distributed in fibrils. Our results showed that in the silk fibers produced by the mutant, P25 broke away from the fibroin units and fibrils, and accumulated in the connecting layer between the silk core and the outer sericin (Fig. 2). We demonstrated that P25, a major component of the ancient cocoon silk structure, is substitutable, as also demonstrated by a recent report of knocking out the P25 coding gene in *Bombyx mori*^{8,31}. However, SER's silk fibers exhibit greater advantages in deep processing and alkali corrosion resistance than WT silk fibers (Fig. 3), and can prevent damage to the silk core fibrils. The P25 protein is distributed in the silk fibroin surface layer of the silk core. The silk fiber's textile material advantages remained unchanged, but new characteristics were additionally derived from the changes in the basic silk fibril structure.

Silk fibroin is a hydrophobic fibrous protein whose molecules are connected by disulfide bonds and whose secondary structure mainly comprises β -sheets^{2,9,32}. SER3 protein is a hydrophilic globular protein whose secondary structure is dominated by random coils³³. In the silk fibers produced by the mutant SER silkworms, the level of β -sheets significantly increased, and the levels of random coils and α -helices significantly decreased. Clearly, it is not a direct contribution by β -sheets of SER3 protein but is caused by changes in the protein secondary structure of the silk fiber. Correspondingly, the mechanical properties such as the maximum stress level and Young's modulus of SER silk fibers were also significantly improved, thereby enabling ultra-thin and ultra-dense fabrics to be woven (Supplementary Fig. 3). Our findings demonstrate the practical value of engineering applications. Moreover, the sericin microsomes dispersed in the fibrils significantly improved the moisture absorption and liberation of the silk fibers, thereby improving the performance of the textile material.

Efficiency of transgene-specific expression of foreign proteins in SGs of *Bombyx mori*. Since the piggyBac transposon-based expression system was developed in silkworms³⁴, dozens of transgenic silkworms with SG expression of foreign proteins have been established. However, the output of these

foreign proteins is far lower than that of cocoon silk, and the higher the molecular weight of the foreign protein, the lower the output. Subsequently, researchers have made breakthroughs in increasing the expression levels of foreign proteins through continuous optimization. For example, with the TALEN-mediated gene replacement system and transgenic technology, the spider's Major ampullate spidroin-1 gene (*MaSp1*) has been used to replace the silkworm *Fib-H* gene; after targeted integration into the PSG for expression, as much as 35.2% of the chimeric protein MaSp1 was obtained from cocoon silk fibers²³. Related explorations have included the introduction of more than three foreign genes into the silkworm genome³⁵ and the use of enhancer combinations (*hr3/IE1*)³⁶. Our laboratory has designed the artificial coding sequence Hpl, which is similar to *Fib-H*, and is specifically expressed in the PSG and binds *Fib-L* more strongly. In the cocoon silk produced by the transgenic silkworms, the content of the foreign protein HPL is 51.9% and 38.93% of the silk fibroin and cocoon silk, respectively³⁰. Although these studies have significantly improved the expression efficiency of recombinant protein, they remain far from achieving the expression level of endogenous silk protein (Supplementary Table 1).

No ideal solution has been described to address the bottleneck problems that commonly occur in SG target tissue transgenic silkworms, such as reduced viability, abnormal SG development and low silk yield^{29,30}. The growth and development of the SGs and individual mutant silkworms in this study were normal. The weight of the cocoon shell, which reflects the protein synthesis and secretion function of the SG, exceeded that of the WT by 16.8%. The cocoon layer rate, which reflects the comprehensive production capacity of mature larvae, was 14.7% higher than that of the control (Supplementary Fig. 2). We demonstrated that while suitable exogenous protein was expressed efficiently, the protein synthesis and secretion ability of the silkworm SG were further improved.

In conclusion, we report an effective silkworm SG transgenic strategy. By selecting non-fibrous protein targets recombinantly expressed by the PSG, the metastable state of the silk protein aqueous solution in the SG cavity was affected, thus enabling alteration of the composition, structure and performance of the fibril molecules of the ancient silk fiber. The mutant completely overcame bottlenecks such as decreased viability, abnormal SG development and low silk yield. Although the suitable SG transgene target proteins remain unclear, the results of this article provide a biological platform for effective in-depth analysis of efficient specific silk protein synthesis by SG cells in the regulation of the synthesis of other proteins. This initial research may provide new ideas for bottom-up molecular design and biological production of silk protein materials.

Materials And Methods

Experimental animal preparation. The classic genetic strain N4W was used in this study. Larvae were reared on fresh mulberry leaves. The entire generation was maintained at 25.0 °C ± 2.0°C in a natural light environment, except for special treatment methods. According to the steps described in Supplementary Text 1, the full-length sequence (3120 bp) of the outer sericin SER3 gene specifically expressed in the MSG was cloned (Supplementary Sequence 1). The strategy in Fig. 1c and steps in Supplementary Text 1 were used to construct the TALEN transgene vector and perform egg injection. The

strategies and effects of mutant screening and genetic purification are shown in Supplementary Fig. 1, and the recombinant SER3 gene insertion site of the mutant was analyzed by tail-PCR sequencing (Supplementary Fig. 1g).

Microscopic observation. The middle cocoon shell and degummed silk fiber of silkworm cocoons were observed by SEM. The spraying current was 20 mA, with platinum vacuum spraying for 3 min. Samples were observed by SEM (S4800, Hitachi, Japan) at room temperature, with repeated observations for three independent samples. The silk fiber was degummed and boiled in 0.2% Na₂CO₃ solution for 30 min.

TEM was used to observe the raw silk samples and SG tissues. The samples were pre-cooled at 4°C and fixed with electron microscope fixative (G1102, Servicebio, China) for 2 h, then fixed with 1% osmium acid for 2–4 h. The fixed samples were dehydrated with an ethanol gradient (50%, 70%, 80%, 90%, 95% and 100%) at 4°C and then dehydrated with 100% ethanol and 100% acetone two times, with each dehydration lasting 15 min. After embedding and sectioning (thickness 60–80 nm), uranium-lead double staining (2% uranyl acetate saturated ethanol solution and lead citrate) was performed for 15 min each, and samples were dried at room temperature, then observed by TEM (HT7700, Hitachi, Japan).

P25 immunofluorescence assays. Paraffin sections of cocoon silk fiber were made according to the conventional method. After dewaxing, sections were soaked in 0.01 M citrate buffer at 96° C for 15 min for antigen repair, and the sections were exposed to blocking solution for 40–60 min. P25 antibody was added to the tissue surfaces of the sections and incubated at room temperature for 1 h. The sections were washed three times with PBST for 5 min each. Then a TRITC labeled secondary antibody (S0015, Affinity Biosciences, Ohio, USA) was added, incubated at room temperature for 1 h, then washed with PBST in the dark three times for 5 min each. Red fluorescence was observed with a fluorescence microscope (BX51, Olympus, Tokyo, Japan).

Secondary structure analysis of silk fibroin. The infrared absorption spectrogram (wavelength range 4000–800 cm⁻¹) of degummed silk fibers was determined with an infrared spectrometer (Nicolet 5700, Thermo Electron Corporation, USA) with a resolution of 8 cm⁻¹. Each sample was scanned 256 times, and three samples were repeatedly analyzed. Spectral data were analyzed in OMNIC 9 software (Thermo Scientific) and PeakFit software (Seasolve, version 4.12). The amide I region deconvolution spectrum fitting method was used, and the peak position was determined by the second derivative peak position of the infrared spectrum.

Silk fiber performance measurement. The mechanical properties were measured with a universal material testing machine (3365, Instron, USA) in a room with constant temperature and humidity (20 °C, R.H. 65%). The test conditions were as follows: initial length, 250 mm; tensile speed, 250 mm/min. The sample was a cocoon silk fiber (100–200 meters) without the sericin protein of the outer layer removed (n = 20 cocoons).

Moisture absorption testing. After degumming, the silk fibers (n = 3 samples) were dried to a constant weight at 80°C and then returned to normal temperature (20°C) for accurate weighing. Hygroscopic

properties were determined in a room with constant temperature and humidity ($20\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$, R.H. $65\% \pm 3\%$), and the weight was measured every 10 min until the fiber reached moisture absorption balance. When the moisture release performance was measured, the sample was first placed in an R.H. 100% container and sealed for 24 h, so that the fiber achieved moisture absorption balance (W_0). Then the fibers were placed in a constant temperature and humidity chamber ($20\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$, R.H. $65\% \pm 3\%$), and the quality changes were monitored continuously until the fiber reached a moisture balance. Regaining of moisture was expressed as the percentage of the mass of water absorbed or released by a unit mass of silk fiber in different time periods with respect to the original fiber mass. The moisture absorption rate and moisture release rate are expressed as water mass absorbed or released by the silk fiber per unit mass at a certain time, according to a previously described method³⁷. Origin2018 software was used to calculate the correlation constants of the fitting curve equation of regaining of moisture over time.

Biocompatibility assays. The degummed silk fibers and non-absorbing polyester suture (NASS) were sterilized under high temperature and high pressure ($121\text{ }^{\circ}\text{C}$, 30 min). L929 (ZQ0093, ZQXZ Biotech, Shanghai, China) mouse fibroblasts were used for cytotoxicity testing. L929 cells were cultured overnight in 96 well plates with 100 μL Eagle's minimum essential medium (ZQ301, ZQXZ Biotech, Shanghai, China). The test fiber (1.0 mg /well) was soaked in the medium and gently cultured in the wells, and the normal cultured L929 cells were used as a negative control. After continuous culture for 24 h and 48 h, a Live-Dead Kit (I3224, Thermo Fisher Scientific, USA) was used to distinguish living cells from dead cells, and an MTT Kit (C0009, Beyotime, Nantong, China) was used to detect cell proliferation. RAW264.7 cells (ZQ0098, ZQXZ Biotech, Shanghai, China) were used for cell inflammatory testing. The cells were cultured in 500 μL Dulbecco's modified Eagle medium (high glucose) (ZQ101, ZQXZ Biotech, Shanghai, China) on a 24 well plate overnight (the number of cells was as high as 3×10^4). The test fiber (10.0 mg/well) was soaked in the culture medium and gently cultured for 24 h and 48 h. The nitrous oxide content in the culture medium was determined with a Nitric Oxide Colorimetric Assay Kit (NO Kit) (S0021, Beyotime, Nantong, China).

Gene expression analysis. An RNAiso Plus (TaKaRa, Dalian, China) was used to extract total RNA from PSG tissues of silkworm larvae on the third day of the 5th instar (5L3d). The cDNA was synthesized with a PrimerScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China) according to the manufacturer's instructions. qRT-PCR was performed in a total reaction volume of 20 μL with the fluorescent dye SYBR Premix Ex Taq (TaKaRa, Dalian, China), according to the manufacturers' instructions, and detected with ABI Stepone Plus (Ambion, Foster City, CA, USA). The *BmRpf49* gene was selected as the internal control. Primers used in this study are listed in Supplementary Table 4.

Western blotting. Silk protein in PSG tissue of 5L3D silkworm larvae was extracted with RIPA lysis buffer (P0013C, Beyotime, Nantong, China) (containing 1 mmol/L PMSF). The total protein concentration was measured with a BCA Protein Assay Kit (Beyotime, Shanghai, China). Western blotting was performed according to conventional methods³⁰. A 100 μg mass of total protein was electrophoresed by 10% SDS-PAGE and then transferred to a PVDF membrane. After blocking at $25\text{ }^{\circ}\text{C}$ for 2 h, HRP-labeled goat anti-rabbit IgG (Bioworld Technology, Minneapolis, MN, USA) was added and incubated. Under dark

conditions, 1 mL EZ-ECL chemiluminescence reagent was added to the membrane, and the bands were observed through chemiluminescence detection (1708370, Bio-Rad, USA) after 1 min.

Data availability

All data generated in this study are available within the Article, Supplementary Information and Source Data files. Source data are provided with this paper.

Declarations

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Author contributions

S.X., X.C., Y.W., and Y.W. designed research; X.C., Y.W., Y.W., Q.L., X.L., and G.W. performed research; S.X., Y.S., and Y.W. contributed new reagents/analytic tools; X.C., and Y.W. analyzed data; and S.X., C.X., and Y.W. wrote the paper. All authors have read and approved the manuscript.

Competing interest

The authors declare no competing interest.

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Figures

Figure 1

Construction of transgenic silkworms. (a) Schematic diagram of SGs producing cocoon silk. The silk core is an ultra-long fiber with a fibril structure, and the outer layer comprises sericin protein synthesized by the MSG. Fibrils are long chains formed by Fib-H/Fib-L/P25 polymer fibroin units synthesized by the PSG. The silk fibroin heavy chain protein (Fib-H), silk fibroin light chain protein (Fib-L) and P25 protein synthesized by PSG cells are secreted into the lumen as fibroin units and then are transferred to the MSG as a metastable high-concentration aqueous colloid. In the MSG lumen, the aqueous colloid of silk fibroin is surrounded by the SER I sericin protein, which is secreted by the back end and middle part of the MSG, and then is surrounded by the sericin proteins SER II and SER III (i.e., SER3), which are secreted by the anterior part of the MSG. (b) Technical strategy. Efficient transgenic expression of SER3 recombinant protein in silkworm PSG cells, to achieve secretion into the PSG lumen and to incorporate water-soluble SER3 into the silk fibroin colloidal solution, alter the metastable state of silk fibroin protein polymers and further affect the silk fiber structure after self-assembly. SM, sericin microsomes expressed in the PSGs and incorporated into the cocoon silk fibrils. Vac, vacuoles in SM. SER, transgenic mutant system for expression of the SER3 recombinant gene in the PSG. WT, wild type. (c) Transgenic piggyBac vector. To enhance the expression and secretion of SER3 protein by PSG cells, the Fib-H gene promoter sequence and 1416 bp of its base sequence containing the signal peptide were introduced upstream of the Ser3 gene sequence with a length of 3120 bp (Supplementary Sequence 1). The EGFP reporter gene sequence

and the 333 bp base sequence at the 3' end of the Fib-H gene were connected downstream of the SER3 gene sequence. Moreover, an artificial promoter, 3 × P3, composed of three tandem PAX-6 transcription factor binding sequences, was specifically expressed in the silkworm eyes and nervous system, and was used to regulate the RFP reporter gene.

Figure 2

Distribution of SER3 protein synthesized by the PSG in cocoon silk and its effect on fiber structure. (a) EGFP fluorescence localization of SER3 protein synthesized by the PSG in silk fiber. (b) Transmission electron micrograph of a longitudinal silk fiber section. SER3 protein synthesized by the PSG is dispersed in the fibrils of the silk fiber. SF, fibroin layer of silk. SS, sericin layer of silk. SM, sericin protein microsomes in the silk fibroin fibrils. Vac, vacuoles. (c) Sericin content of cocoon silk. (d) Immunofluorescence localization of P25 protein in silk fiber. WT-L and SER-L, longitudinal section of silk fiber; WT-C and SER-C, cross-section of silk fiber. (e) Deconvolution of amide I bands in silk fibers, analyzed by FITR. The amide I band (1700–1600 cm⁻¹) was deconvoluted with the Fourier self-deconvolution method to determine the changes in silk fiber β -sheets, random coils and α -helices. The black solid line is the amide I band spectrum, and the dotted line is a separate deconvolution peak. Peak abbreviation mark: T, β -turn; A, α -helices; R, random coil, B, β -sheets, SC, side chain. (f) Statistics of protein secondary structure components in silk fibers.

Figure 3

The SER3 protein secreted by the PSG improves the mechanical properties and the moisture absorption and liberation of silk fibers. (a-h) Fiber mechanical properties. The monofilament extracted from cocoons was boiled with 0.2% sodium carbonate for 30 min to remove the outer sericin protein and obtain textile fibroin fiber. (a) Stress and strain curve. (b) Stress level. (c) Modulus of elasticity. (d) Young's modulus. (e) Moisture absorption rate (constant temperature and humidity conditions: 20 °C $\pm$ 2 °C, R.H. 65% $\pm$ 3%).

(f) Moisture absorption speed. The fitted curve equations of WT and SER fibroin fiber samples are $v=12.276-12.163e^{-0.0756t}$, $R^2=0.9966$; $v=13.470-13.370e^{-0.0980t}$, $R^2=0.9954$. t, Time. (g) Moisture liberation rate (constant temperature and humidity conditions: 20 °C ± 2 °C, R.H. 100%). (h) Moisture release speed. The fitted curve equations of WT and SER fibroin fiber samples are $v=12.353+17.619e^{-0.03381t}$, $R^2=0.9977$; $v=13.184+23.552e^{-0.04187t}$, $R^2=0.9990$. t, Time. (i & j) Scanning electron microscopy (SEM) characterization of cocoon (i) and fibroin fiber (j). (k-n) Cytotoxicity and inflammation testing. Fibroin mixed culture cells for 48 h. (k) Cell morphology, assessed by Live-Dead staining and (l) proportion of dead cells. (m) Relative proliferation rate of L929 cells, detected with the MTT method. (n) Content of nitric oxide in the medium of RAW264.7 cells. Null, control. WT, fibroin of WT. Ser, fibroin of SER; NASS, medical non-absorbable suture. **, $P<0.01$.

Figure 4

Synthesis and secretion of silk proteins in SGs of mutant 5th instar larvae. (a) Frozen section of the SG cross-section. The state of the SER3 protein secreted into the lumen of the SG, detected by EGFP-fusion expression. (b) Transmission electron micrograph of the PSG. Abbreviations in Fig. 4a and 4b: SF, silk fibroin layer; SS, silk sericin layer; er, endoplasmic reticulum; G, Golgi apparatus; m, mitochondrion; mf, fibroin mass; mv, microvilli. (c) Semi-quantitative PCR and (d & e) qRT-PCR to detect the mRNA levels of EGFP, SER3, and silk fibroin Fib-H, Fib-L and P25 genes in cells in different parts of the SG. MA, MM and MP show the anterior, middle and posterior parts of the MSG, respectively. PA and PP show the anterior and posterior parts of the PSG, respectively.

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