Honeybee silk: Recombinant protein production, assembly and fiber spinning

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\textbf{A B S T R A C T}

Transgenic production of silkworm and spider silks as biomaterials has posed intrinsic problems due to the large size and repetitive nature of the silk proteins. In contrast the silk of honeybees (Apis mellifera) is composed of a family of four small and non-repetitive fibrous proteins. We report recombinant production and purification of the four full-length unmodified honeybee silk proteins in \textit{Escherichia coli} at substantial yields of 0.2–2.5 g/L. Under the correct conditions the recombinant proteins self-assembled to reproduce the native coiled coil structure. Using a simple biomimetic spinning system we could fabricate recombinant silk fibers that replicated the tensile strength of the native material.

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\textbf{1. Introduction}

Silks are protein materials produced by a wide range of insect and spider species for applications requiring high-performance fibers. The silk of the domesticated silkworm, \textit{Bombyx mori}, has been used as a suture biomaterial for centuries, and in recent years farmed silkworm silk has also been reprocessed into forms such as films, gels and sponges for medical applications [reviewed in 1]. In order to further expand the field of applications for the versatile silk materials, there has been much interest in recombinant production of silk proteins. However, numerous efforts to clone and express either silkworm silk or the remarkably strong spider dragline silks in transgenic systems have found it challenging. The large size (> 10 kbp) and highly repetitive sequences of these silk genes make them recalcitrant to expression outside specialized silk glands, and lead to low protein yields [2].

Although silkworm cocoons and spider webs are the best known silks, other insect species produce silks that may be better suited to transgenic production. Of particular interest is the cocoon silk spun by honeybee larvae (\textit{A. mellifera}), which has been shown to increase the mechanical strength and thermal stability of the beehive [3]. The honeybee silk is encoded by four small (\sim 1 kbp each) and non-repetitive fiber genes [4]. The small size and low level of repetition in these sequences makes them excellent candidates for transgenic silk production. Homologous sets of four genes have been found in bumblebees, bulldog ants, weaver ants, hornets and Asiatic honeybees, with each of the gene products present in the mature silk [5–7]. The conservation of each of the four homologous genes in ants and hornets – species which diverged from the bees over 150 million years ago - implies that the gene products fill distinct functional roles in the silk material.

Vintage x-ray fiber diffraction work demonstrated that honeybee silk contains \textit{\alpha}-helical proteins assembled into a higher order coiled coil conformation [8]. A more detailed study indicated a tetrameric coiled coil [9]. As the four silk proteins of honeybees are expressed at approximately equal levels, they likely correspond to the four strands of the coiled coil structure [5]. Bioinformatics techniques predict that each of the honeybee silk protein sequences contains 60–68\% coiled coil [4]. A recent NMR study reported that honeybee silk proteins have both \textit{\alpha}-helix and \textit{\beta}-sheet structures, but confirmed that the \textit{\alpha}-helical conformation predominates [10].

Honeybee silk is produced from modified salivary glands known as labial silk glands. Within the gland the secreted silk proteins assemble into cigar-shaped bodies a few microns long, which are strongly birefringent indicating an oriented structure [11]. The protein concentration in honeybee silk glands is not known, but is probably similar to the 25–30\% found in silkworm and spider glands [12]. Silk threads can be hand-drawn from
the silk glands of honeybee larvae. These threads have greater extensibility and toughness than silkworm silk fibers with lower tensile strength [13].

A recent paper reported recombinant production of Asiatic honeybee silk (Apis cerana) [7]. The four A. cerana silk proteins were expressed in a soluble form in E. coli with yields of 10–60 mg per liter of ferment. Circular dichroism, NMR and light scattering techniques were used to characterize the structure and interactions of the proteins at low concentration (0.03–0.2 wt%). These conclusively demonstrated that neither the individual proteins nor a mix of four proteins had tight tertiary packing in solution in the concentration range measured. The proteins existed as monomers or loosely associated dimers and had predominantly random-coil conformation. This shows that producing dilute recombinant silk protein solutions and combining them is insufficient to induce the correct secondary and tertiary structure of native honeybee silk.

In this work we optimize heterogeneous expression conditions to obtain high yields of recombinant A. mellifera silk proteins that are amenable to correct folding. We characterize the silk proteins in highly concentrated solution, as found in the honeybee silk gland, and in the solid state, as found in the mature honeybee silk product. We draw recombinant silk threads and test their mechanical properties at a proof of principle level, to enable consideration of the applications of honeybee silk as a biomimetic material.

2. Materials and methods

2.1. Recombinant protein production

The four honeybee silk fibrin proteins (Amef1-4) are 30–34 kDa in size and each contains an ~21 kDa central region of coiled coil motifs [4]. The proteins are related but highly diverged, with 24–31% amino acid identity [4]; a sequence alignment is shown in Supplementary Data Fig. 1.

To create recombinant expression constructs, the four honeybee silk gene sequences (FJ235088, FJ235089, FJ235090, FJ235091) without signal peptides were subjected to PCR amplification using the following primer sets: Amef1F1: GGAATT C TCT TGC TAC ATG TAT TTT C.

2.2. Protein purification and solubilization

Cells were resuspended in approximately five times their volume of BugBuster Master Mix (Novagen) and incubated for 30 min. The inclusion bodies were harvested by centrifugation at 10,000 g for 10 min. The pellet was washed in approximately five times its volume of BugBuster reagent, then twice in approximately six times its volume of BugBuster reagent diluted tenfold. The protein composition of the inclusion bodies was analyzed after protein separation by SDS-PAGE on 4–12% gradient bis-tris gels (Invitrogen). Intensity of protein bands was measured by the program ImageQuant TL (GE Healthcare). Recombinant silk protein identification was verified by tandem mass spectrometry as previously described [4].

Inclusion bodies were solubilized in 3% sodium dodecyl sulfate (SDS) with 2 h incubation at 60 °C. Protein concentration in solution was measured using a QuantiTec BCA assay kit (Sigma). Where required, solutions of each of the four recombinant honeybee silk proteins were mixed at equimolar ratios. Excess SDS was removed from protein solutions by dialysis against 5 g/l KC1 solution causing precipitation of the potassium salt. The precipitate was removed by centrifugation at 16 000 g for 5 min and was dried and weighed to determine the amount of SDS removed.

2.3. Fiber spinning

Recombinant silk protein solutions were concentrated by extended dialysis against 20 wt% polyethylene glycol (PEG, MW 8000, Sigma) or Slide-A-Lyzer concentrating solution (Pierce), until a honey-like viscosity was obtained. Droplets of concentrated silk dope (~40 μl) were suspended between the prongs of pairs of tweezers in air and the tweezers were opened to draw fine fibers. Fibers were mounted on frames using epoxy glue and dried in air. After drying the frames were cut, the fibers were submerged in a 90% methanol 10% water bath, drawn a second time to approximately 2x length, and air-dried.

2.4. Fiber characterization

Single-drawn and double-drawn recombinant silk fibers were examined by a polarized light microscope and by a Zeiss EVO LS15 environmental scanning electron microscope at 15 kV voltage and 10 Pa pressure. Single-drawn and double-drawn fibers and recombinant silk films were analyzed by wide-angle X-ray scattering at the SAXS/WAXS beam line of the Australian Synchrotron. A wavelength of 0.0886 nm and camera length of 0.558 m provided a q-range of approximately 0.7–14 nm⁻¹, which was calibrated using a silver behenate standard.

Tensile tests of single-drawn and double-drawn fibers were carried out on an Instron Tensile Tester model 4501 at a rate of 2.5 mm/min. Tests were conducted in air at 21 °C and 65% relative humidity. Prior to testing each fiber was placed across a 3 mm slot in a plastic frame and fixed with epoxy glue. The gauge length and diameter of each fiber were measured by optical microscopy.

2.5. Fourier transform infrared spectroscopy (FTIR)

Native honeybee silk sheets were obtained from a commercial hive, washed extensively in chloroform to remove wax and washed extensively in warm water to remove water-soluble contaminants. Solutions of each of the four recombinant honeybee silk proteins were mixed at equimolar ratios, cast and dried into solid films. Some air-dried films were later submerged in a 90% methanol bath for 2 min and then dried. Purified inclusion body samples from each of the four protein expression systems were dried into solid films. Infrared spectra from these samples were obtained in transmission mode using an i-series imaging infrared microscope accessory. Spectra were collected using Spectrum software (version 5.3.1) and represent the average of 256 scans collected at a resolution of 4 cm⁻¹. Post-collection data manipulation and analysis was carried out using Grams/Al software v5.05.

3. Results

3.1. Production of recombinant honeybee silk proteins in inclusion bodies

The four honeybee silk proteins, Amef1-4, were successfully synthesized in recombinant E. coli cells at laboratory scale. SDS-PAGE gels demonstrated that the silk proteins were expressed in the soluble fraction at 20 °C incubation temperature and in the
insoluble fraction at 30 and 37 °C incubation temperatures. The highest yields of recombinant protein were obtained when expression was conducted for extended periods (24–36 h) at temperatures ≥30 °C with the proteins recovered from the inclusion bodies. Quantitative intensity analysis of SDS-PAGE gels (Fig. 1), with protein identities confirmed by mass spectroscopy, indicated that the protein component of the inclusion bodies was essentially pure (>95%) silk protein. As subsequent analysis (described below) found that proteins solubilized from inclusion bodies self-assembled into native-like structure, all heterologous expression was conducted under conditions such that recombinant proteins were recovered from the inclusion bodies.

In order to produce substantial quantities of silk proteins a large-scale fermentation process was developed and optimized for expression of the silk protein AmelF3. Use of rich medium (Luria broth) was compared to minimal medium, using different carbon sources (glucose and glycerol) and inducers (lactose and IPTG). Optimal protein expression was obtained in a combination of batch and fed-batch fermentation using minimal medium with glucose as the initial carbon source then switching to glycerol following induction with IPTG. Under these conditions the OD_{600nm} value of the ferment was 34 and the yield of purified recombinant AmelF3 after solubilization (described below) was approximately 2.5 g per liter of ferment. The same fermentation conditions were used to express the other honeybee silk proteins. The strains expressing silk proteins AmelF1, 2 and 4 grew to OD_{600nm} values of 30, 67, and 57 respectively. The yields of purified recombinant proteins AmelF1, 2 and 4 after solubilization (described below) were approximately 0.2, 1.5 and 1.9 g per liter of ferment respectively.

### 3.2. Protein solubilization and refolding

Purified inclusion bodies, each containing a single recombinant honeybee silk protein, were analyzed by FTIR and the protein structures within the inclusion bodies were found to be misfolded into predominantly β-sheet conformations (not shown). The misfolded proteins in the inclusion bodies were solubilized in 3% SDS to
give silk protein solutions of between 0.5 and 3 wt.% protein. As SDS is not a desired component of a mature silk product and disrupts protein tertiary structure, excess SDS was removed from the protein solutions by adding KCl. The potassium salt of dodecyl sulfate (KDS) has low solubility (around 0.2%) and therefore precipitates out of solution, where it can be removed by centrifugation. Protein concentration measurements indicated that a small quantity of protein co-precipitated with the KDS salt, with protein loss measured as 6–10%. Weighing the KDS precipitate indicated that the potassium treatment removed 70–80% of the detergent from the protein solutions.

FTIR spectra were obtained from three samples of solid honeybee silk. The first sample was a native silk sheet washed with chloroform to remove wax (Fig. 2A). X-ray scattering analysis indicated that the chloroform wash did not alter the native protein structure (Supplementary Data Fig. 2). The second sample was an equimolar mix of the four purified recombinant silk proteins that was dried into a film (Fig. 2B). The third sample was a recombinant silk film identical to the second sample that was additionally treated by submersion in a 90% methanol bath (Fig. 2C). The deconvolution of the amide I region for the recombinant and native silk spectra is shown in Fig. 2, and a summary of the results and the component secondary structure assignments is presented in Table 1. Assignment of peaks to coiled coil structure is based on the characteristic three-band fingerprint observed in coiled coil spectra [14]. The FTIR results suggest that the native honeybee silk contains approximately 65% coiled coil structure. The air-dried recombinant silk spectrum is very similar to the native silk spectrum, and the recombinant silk is estimated to contain 59% coiled coil structure. The broad-range FTIR spectrum of air-dried recombinant silk contains an SDS peak about half as strong as the protein amide I band (Supplementary Data Fig. 3); however, the presence of this quantity of SDS apparently does not hinder coiled coil formation. The methanol-treated recombinant silk spectrum has a strong peak at 1621 cm\(^{-1}\) that is not present in the native silk and is attributed to beta-sheet structure. The estimated level of coiled coil structure in the methanol-treated silk has dropped to 48%.

### 3.3. Spinning dope preparation

The recombinant silk solutions were concentrated by dialysis to levels approaching those expected in insect silk glands [12]. During the concentrating stage, single silk protein solutions began to precipitate at 2–4 wt.% protein. Precipitation could be prevented by addition of SDS; however, threads could not subsequently be drawn from concentrated droplets of these solutions. Conversely, silk solutions containing an equimolar mixture of the four silk proteins did not precipitate during the early stages of dialysis. They could be concentrated to 10–15 wt.% protein, at which point the dope became brown in colour and honey-like in consistency. This dope was stable at room temperature for up to two days or at 4 °C for more than three months.

### 3.4. Drawing and characterization of recombinant silk fibers

Silk dope was hand-drawn in air to form fine threads (Fig. 3A). These single-drawn fibers were stable in air but were soluble in water, unlike the native silk. The threads were submerged in a 90% methanol bath and redrawn to approximately twice their original length during which they changed in appearance from opaque to translucent. The double-drawn threads were finer (Fig. 3B) and were stable in aqueous solution.

Recombinant silk threads imaged by ESEM (Fig. 3C and D) were circular in cross-section and uniform in diameter along their length. Single-drawn fibers had small bodies adhering to their surface that could be residual salt crystals (Fig. 3C), however double-drawn fibers had smooth surfaces (Fig. 3D). Polarized light microscopy showed that single-drawn fibers are not birefringent, but that double-drawn fibers are strongly birefringent (Fig. 3A and B), suggesting high protein alignment.

Recombinant silk threads and a recombinant silk film were examined by wide-angle x-ray scattering. The WAXS patterns for the film and for single-drawn fibers (Supplementary Data Fig. 4A) were dominated by a strong signal from residual SDS crystals, but this was not detectable in the double-drawn fibers (Supplementary Data Fig. 4B). We therefore calculate that double-drawn threads contain <0.1% of the SDS crystals per unit length found in the single-drawn threads, so the final material is effectively SDS free. The protein scattering patterns from recombinant silk could not be analyzed due to low scattering signal from the very fine fibers.

The strength and extensibility of recombinant honeybee silk threads were measured by standard tensile testing techniques. Due to the innate variability of hand-drawing techniques together with the difficulties of handling very fine fibers, a number of the recombinant threads contained flaws and readily broke at the damaged points. We avoided analyzing flawed and thus non-representative fibers by excluding the weaker half of the tested fibers from our sample. Table 2 presents thread diameter and tensile data for the strongest ten double-drawn fibers (from 21 fibers tested) and the strongest three single-drawn fibers (from six fibers tested). The table also compares the mechanical properties of recombinant silk fibers to the properties of native fibers drawn from the honeybee silk gland. Stress-strain plots of representative single-drawn and double-drawn fibers are shown in Fig. 4.

### 4. Discussion

In this work we have achieved a yield of 2.5 g/L purified full-length silk protein, which is the highest reported expression level for any recombinant silk protein. For comparison, a previous high

### Table 1

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<th>Assignment</th>
<th>Native honeybee silk sheet washed in chloroform</th>
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<th>Recombinant honeybee silk film treated in 90% methanol</th>
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was the production of a partial-length synthetic spider silk at a yield of 0.36 g/L [15]. Two factors have contributed to the high yields that we observe. First was a careful selection of our target silk system. In contrast to the large size (>10 kbp) and highly repetitive nature of the genes encoding the well-studied dragline silk of spiders and cocoon silk of silkworms, the honeybee silk genes are small (approximately 1 kbp) with far less repetition in their DNA sequences [4]. The smaller size and reduced level of repetition means that the honeybee genes are not prone to the genetic instabilities including premature translation termination and truncation that result from transgenic expression of highly repetitive nucleotide sequences. Secondly we developed a novel approach to protein expression and purification, which may be broadly adaptable to other fibrous proteins. A fermentation system was optimized for maximum segregation of recombinant proteins into *E. coli* inclusion bodies rather than the soluble fraction. The inclusion bodies were purified in a single step to yield high-purity silk proteins without need to include a tagging sequence. The proteins were solubilized using SDS, and then most of the detergent was removed by precipitation. Our WAXS data showed that the remaining SDS was subsequently removed during the final stage of the fiber drawing process.

Analysis of FTIR data (Table 1) suggested that the native honeybee silk contains approximately 65% coiled coil structure, which is consistent with previous sequence–based predictions [4] and NMR results [10]. Even in the presence of some SDS, an equimolar mixture of the recombinant silk proteins was found to be refolded into a material containing ~59% coiled coil, which closely mimics the native structure. Methanol-treated recombinant silk was less like the native sheets, with an estimated 48% coiled coil, as methanol exposure apparently caused a partial structural transition from coiled coil to beta-sheet material.

The honeybee silk contains four related fibrous proteins. Homologues of the four honeybee fibroin genes encoding these proteins have been conserved in ant and wasp species that have been separated from the bees by over 155 million years of evolution.
implying that the gene products are functionally non-redundant in the silk. In our experimental system, single recombinant proteins were less stable in solution than an equimolar mixture of the four proteins and could not be concentrated enough to obtain a silk dope with sufficient viscosity for thread formation. Thus the coexistence of four proteins may be linked to maintaining protein solubility at high concentrations in the silk gland. We hypothesize that the four proteins form a tetrameric assembly that effectively shields hydrophobic residues from water, averting hydrophobic aggregation and precipitation.

It was previously demonstrated that honeybee silk fibers could be hand-drawn from the contents of the silk gland [8], and the resultant fibers possessed aligned coiled structure and good mechanical properties. In the same manner we were able to hand-draw fibers from a sufficiently concentrated and viscous mixture of the four recombinant silk proteins (Fig. 3C). Although the silk dope used to generate these threads was expected to self-assemble into coiled coil structures when dried (Fig. 2B), the air-dried threads were not birefringent (Fig. 3A), suggesting that the proteins were not aligned. Possibly the proteins were aligned by the shear forces during draw, but had time to relax into a non-aligned conformation before they dried. The single-drawn fibers had comparable extensibility to native silk fibers but had low tensile strength (Table 2). A second draw of the fibers in a methanol bath, combining shear and rapid dehydration, was more successful in aligning the proteins, as the double-drawn fibers reproduced the native birefringence (Fig. 3B). The second drawing process reduced fiber extensibility; however the double-drawn fibers slightly exceeded the tensile strength observed in the native fibers (Table 2).

The simple protein spinning system described in this work was able to produce recombinant fibers which mimicked either the strength or the extensibility of native honeybee silk fibers but not both simultaneously. A study of honeybee silk gland contents prior to spinning observed many parallel cigar-shaped bodies with high birefringence [11], suggesting that proteins within the gland are pre-assembled into aligned liquid crystals before initiation of draw. Our group is now working to develop an alternative fabrication technique that will pre-align the silk dope prior to spinning.

5. Conclusions

Full-length unmodified recombinant honeybee silk proteins can be produced at remarkably high yield and purity in inclusion bodies by E. coli fermentation. Recombinant proteins self-assemble into a coiled coil structure similar to that observed in native honeybee silk. The recombinant proteins can be fabricated into silken threads as strong as threads drawn from the honeybee silk gland. This work is a significant step towards development of coiled coil silk biomaterials.

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Appendix. Supporting material

The supplementary data associated with this article can be found in the on-line version at doi:10.1016/j.biomaterials.2009.12.021.

References