Design, Expression, and Structural Characterization of Hybrid Proteins of *Samia cynthia ricini* and *Bombyx mori* Silk Fibroins

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ABSTRACT: Genetically engineering strategies have been applied to obtain the designed proteins [GGAGSGYGG-GYGHGYGSDGG(GAGAGS)₃]_n of varying chain lengths ($n = 2, 4$ or 6); SLP2, SLP4, and SLP6, respectively. The amino acid sequence of the monomeric unit represents the incorporation of the consensus repeat sequences of the glycine-rich region of *Samia cynthia ricini* followed by the repetitive crystalline region of *Bombyx mori* silk fibroins. The oligonucleotides were designed to encode the desired monomeric unit of silk-like hybrid proteins, which were then polymerised and expressed in *Escherichia coli* system. The monomer, SLP1, was synthesized chemically using solid phase method. The recombinant proteins were identified by N-terminal amino-acid sequencing and Western blotting. The structural characterizations of SLP1 and SLP6 samples were performed using ¹³C CP/MAS NMR in the solid state. The structure of SLP1 in the solid state was distorted β -turn by judging from the chemical shift and line shape of the C ^{β} carbon of Ala residue, and the structural transition from distorted β -turn to β -sheet occurred partially in SLP6. The solution structure was studied in hexafluoro-2-propanol (HFIP) using CD method. Although both samples, SLP1 and SLP6, showed indication of the ordered structures, marked change in the band intensities was observed between two CD spectra. Especially, the CD pattern of SLP6 is in agreement with the pattern with right-handed 3₁₀-helical conformation.

KEY WORDS Silk-like Hybrid Proteins / Genetically Engineered Proteins / ¹³C Cross Polarization Magic Angle Spinning NMR / Circular Dichroism / *B. mori* and *S. c. ricini* Silk Fibroins /

Natural silks have become the subject of extensive study as model systems for high-performance biopolymers and polymer composites in part because of their impressive mechanical properties of the fibers.¹ In addition to high quality textiles, silks have been recognized to have not only excellent enzyme immobilization properties but also suggested to find potential applications in design and development of various biotechnological and biomedical devices.^{2–10}

The unique amino acid composition and the primary structure of the silk fibroin appear to be important⁹ despite the fact that there are wide variations in the primary and secondary structures among different silks. For example, a well-known fibrous protein from the domesticated silkworm, *B. mori*, exhibits the amino acid composition (in mol%) as 42.9% Gly, 30.0% Ala, 12.2% Ser, 4.8% Tyr, and 2.5% valine.⁹ On the other hand, the amino acid composition of the silk fibroin from a wild silkworm, *S. c. ricini*, is quite different. While the sum of Gly and Ala residues (82%) is similar to *B. mori* (73%) silk, the relative proportions of these residues are reversed (48.4% Ala and 33.2% Gly) in *S. c. ricini*.^{11,12} An analysis of the amino acid sequence of *B. mori* silk fibroin reported by Zhou *et*

al.,¹³ revealed the presence of unusual highly repetitive GAGAGS sequence motifs, comprising about 55% of the entire fibroin molecule whereas, the silk fibroin of *S. c. ricini* has been shown to consist of the relative much longer glycine-rich motifs: GGAGSGYGGGYGHGYGSDGG, also referred as amorphous region, flanked by poly-L-alanine (PLA) regions.^{12,14} The available information points towards that the (GAGAGS)_n motifs may contribute significantly to the high strength of *B. mori* silk fiber. Extensive investigations of naturally produced silk materials have clearly indicated that there exists a strong correlation between the amino acid sequence and conformation-property relationships. Therefore, the production of high quality fibers with distinct mechanical properties would require not only the design of primary structure but also the precise knowledge of their secondary structure. The available information of the amino acid sequence motifs, *i.e.*, the peptide modules, may be valuable. Moreover, the mixing of the modules in different proportions, by genetic engineering methods, would provide a unique opportunity to evaluate and produce the fibers with desired tensile strengths that may find potential medical and biotechnological applications.

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For native silk fibroin from *S. c. ricini* stored in the silk gland or the film prepared from the silk fibroin by drying it gently at room temperature,^{14,15} the PLA regions predominantly take an α -helical structure.^{11,16–18} On the other hand, by stretching the film or immersing it into methanol-water mixture, the structure of PLA changes to β -sheet structures. However, only limited information are available on the glycine-rich regions of *S. c. ricini*.^{12,14} The two well-known crystalline forms of *B. mori* silk fibroins are the silk I (structure in the solid state before spinning) and silk II (structure in the solid state after spinning) forms, often referred as the two dimorphs of the fibroin molecules. Using a simple model peptide (AG)₁₅, the conformational characteristics of the ‘less stable’ silk I form have recently been clarified as a repeated type II β -turn structure (Ala; $\phi_{i+1} \sim -60^\circ$, $\psi_{i+1} \sim 130^\circ$, Gly; $\phi_{i+2} \sim 70^\circ$, $\psi_{i+2} \sim 30^\circ$) stabilized by intramolecular hydrogen bonds.¹⁹ On the other hand, we used ¹³C solid state NMR to clarify the heterogeneous structure of the natural fiber and the crystalline region from *B. mori* silk fibroin with silk II form.^{20,21} The relative proportions of the various heterogeneous components have been determined from their relative peak intensities. The various combinations of specific fibroin sequence motifs are expected to yield variations in the fiber’s properties and may provide an opportunity to design unique biomaterials.

In this study, we attempted to synthesize the silk-like hybrid proteins with defined sequence: [GGAGS-GYGGGYGHGYGSDGG (GAGAGS)₃]_n, which have the primary structure incorporating the Gly-rich motif of *S. c. ricini* at the N-terminus and the highly repetitive motif (GAGAGS)_n of *B. mori* silk fibroin at the C-terminus. If we can prepare the fiber from these silk-like hybrid proteins, the fiber is expected to have different physical and mechanical properties from the original *B. mori* and *S. c. ricini* silk fibroin fibers. This provides us a variety of biomaterials with different properties. Thus, we can find out the biomaterials which are suitable to our individual purpose. In the process of the preparation, it might be possible to find out the fibers with both high strength and high toughness. The structural analyses of the monomeric and hexameric polypeptides, SLP1 and SLP6, respectively, in the solid state, were performed using ¹³C cross polarization/magic angle spinning (CP/MAS) NMR. In addition, the circular dichroism (CD) measurement was made in the hexafluoro-2-propanol (HFIP) solvent system.

EXPERIMENTAL

Materials

E. coli strain DH5 α was used for propagation and construction of plasmids, and *E. coli* strain BL21(DE3)pLysS was used for production of proteins. Synthesized DNA fragments with phosphorylation were purchased from Sigma Genosys, Japan. Restriction enzymes and ligase were purchased from Takara Shuzo, Japan. Plasmid pUC118 was obtained from Takara Shuzo, and pET30a was obtained from Novagen. All other chemicals were of analytical grade. Bacterial growth in rich medium, DNA manipulations, and transformation conditions were performed as described in the reference by Sambrook *et al.*²² DNA sequencing was performed on a ABI PRISMTM 377 Auto Sequencer according to its user’s manual. Cell density was measured on a Hitachi U-3200 spectrophotometer in quartz cuvettes with a path length of 1 cm at $\lambda = 600$ nm. Batch cultures were performed on MDL-6C Fermentor (B. E. Marubishi Co.), with 1.2 L working volume.

Construction of pUC118-linker

Figure 1a shows a synthetic adapter containing *Xba* I, *Spe* I, and *Nhe* I restriction enzyme sites, which was ligated into pUC118 that had been digested with *Xba* I and dephosphorylated using Calf Intestine Alkali Phosphatase (CIAP), to form the ampicillin resistant pUC118-linker. The ligation mixture was used to transform *E. coli* strain DH5 α with selection on 2 $\times$ YT agar medium containing 50 μ g mL⁻¹ ampicillin. Recombinant plasmid pUC118-linker was examined by double-stranded DNA sequencing with M13 primers M4 and RV. The sequencing reaction was performed according to standard *PE Biosystems* sequencing protocol.

Construction of Monomer Fragment

Four oligonucleotides shown in Figure 1b were annealed to generate two DNA monomer fragments encoding *S. c. ricini* Gly-rich and *B. mori* crystal regions, respectively. The purified duplex DNAs containing the *Bam*H I cleavage sites at both fragments were ligated into the *Bam*H I site of pUC118. The transformation and sequencing were performed in the same manner as described above. After digestion of the recombinant pUC118 containing a monomer fragment with *Nhe* I and *Spe* I, the fragment was ligated into *Nhe* I-*Spe* I site of pUC118-linker.

Peptide Synthesis

The monomeric unit [GGAGSGYGGGYGHGY-GSDGG(GAGAGS)₃AS] was synthesized by solid phase F-moc chemistry on a fully automated Pioneer Peptide Synthesis System (Applied Biosystems Ltd.) as described elsewhere.^{19,21} The peptide was assembled on F-moc-Gly-PEG-PS resin and the coupling of F-moc amino acids were performed. After synthesis, the free peptides were released from the resin by the treatment with a 40 mL mixture of TFA, phenol, triisopropylsilane and water (88:5:2:5% by volume) for two hours at room temperature. The crude peptide was precipitated, washed with cold diethyl ether and dried under vacuum. The precipitate was dissolved in 9 M LiBr and dialyzed extensively against distilled water. The peptide was collected by lyophilization.

Cp Fraction of *B. mori* Silk Fibroin

The Cp fraction of *B. mori* silk fibroin was prepared from regenerated silk fibroin solution as described elsewhere.²¹ Chymotrypsin (40 mg, Seikagaku Kogyo Co., Japan), dissolved in a few mL of water, was added to an aqueous solution of about 4 g of fibroin buffered with Na₂HPO₄·12 H₂O and NaH₂PO₄·2 H₂O at pH 7.8. The solution (200 mL) was incubated at 40 °C for 24 h, and the precipitate that formed (Cp fraction) was separated by centrifuging at 10000 rpm followed by washing with 0.03 N HCl to inactivate the enzyme reaction. Then the precipitate was washed several times with distilled water, ethyl alcohol, and ethyl ether. Finally the precipitate was dried in vacuum, yielding 56% of original fibroin. The structure of this Cp fraction is silk II. The Cp fraction in the silk I form was prepared by dialyzing the Cp fraction in 60% aqueous LiBr against similar solutions of LiBr that were diluted progressively with water. This procedure results in a precipitate with the silk I form.

Solid State NMR Observation

Solid state ¹³C CP/MAS NMR spectra were acquired on a Chemagnetics CMX-400 spectrometer operating at 100 MHz, with a CP contact time of 1 ms, TPPM decoupling, and magic angle spinning at 10 kHz. A total of 10000–25000 scans were collected over a spectral width of 60 kHz, with a recycle delay of 3 s. Chemical shifts are reported relative to TMS as reference.

Circular Dichroism

The solution-state CD spectra (185–260 nm) of SLP1 and SLP6 were recorded in HFIP (Wako Pure Chemical Industries Ltd.) on a JASCO J-805 Spectropolarimeter. A cylindrical quartz cell of 0.01 cm was used for spec-

tral measurements. The spectra were recorded at an ambient temperature and each spectrum is presented as an average of eight consecutive scans measured at 1 nm resolution. The data were processed on a computer with the available software and results are presented as residual molar ellipticity [θ]_M (deg. cm² dmol⁻¹) based on number of residues 38 for monomer and 228 for hexamer, with the molecular weights 2889 and 17334, respectively.

RESULTS AND DISCUSSION

Construction of Monomeric and Multimeric SLP DNAs

In order to produce the tandem repetitive polypeptides, DNA sequences shown in Figure 1b were designed: The 5'-*Bam*H I site at *S. c. ricini* Gly-rich region and 3'-*Bam*H I site at *B. mori* crystalline region are positioned at each end for insertion of the DNA into *Bam*H I site of cloning plasmid pUC118. The *Spe* I and *Nhe* I restriction sites encoding Thr-Ser and Ala-Ser were inserted after and before *Bam*H I sites, respectively, in order to isolate the DNA fragments from the recombinant plasmids. Optimal codons for each amino acid can be selected using codon usage in *E. coli*.²⁴ Since the polypeptide expression level and the stability of the DNA itself are influenced by the base composition and sequence of the DNA, the codons were selected as many as A-T contents as possible. The DNA multimers were inserted into *Nhe* I and *Spe* I digested pUC118-linker, flanked by an ATG codon encoding methionine at each site in order to obtain the periodic portion of SLP by cleavage of the expressed hybrid proteins with cyanogens bromide (CNBr).

Plasmids, pUC118 containing tandem repeats of the synthetic DNA fragments were identified by restriction digestion analysis using *Bam*H I and DNA sequencing. Multimers were formed by insertion of *Nhe* I-*Spe* I fragments into *Nhe* I digested vectors since, these enzymes generate identical cohesive ends, to build, in a tightly controlled fashion, larger genes of any desired degree of multimerization. We have exploited these strategies for the controlled construction of multimers ranging in size from 1 to 6 repeats of the SLP sequence while retaining unique *Nhe* I and *Spe* I sites in the constructs. The constructs of SLP multimers are summarized in Table I. The agarose gel electrophoresis of these constructs after *Bam*H I digestion are shown in Figure 2 and the results are the same as our expected base pairs.

Protein Expression and Analysis

Our efforts for expression utilized the commercially available expression vector pET30a which places the

Figure 2. *Bam*H I digestion analysis of multimerized oligonucleotide sequences. **M**: molecular weight markers; **lane 1**: pUC118-SLP2; **lane 2**: pUC118-SLP4 and **lane 3**: pUC118-SLP6.

Figure 3. Identification of the recombinant hybrid proteins His₆-SLP2, His₆-SLP4, and His₆-SLP6 obtained at various stages of purifications. **a**: hybrid protein His₆-SLP2 produced using pET30a-SLP2 and after Ni-NTA column chromatography, **b**: hybrid protein His₆-SLP4 produced using pET30a-SLP4 and **c**: hybrid protein His₆-SLP6 produced using pET30a-SLP6. The protein bands were visualized by Coomassie staining. **M**: molecular weight markers; **E**: after elution with 250 mM imidazole solution, **CL**: cell lysate, **FT**: flow-through, and **W**: after washing with 20 mM imidazole solution. The hybrid proteins were eluted with 250 mM imidazole solution, which have the molecular weight of 19 kDa (**aE**), 29 kDa (**bE**), and 40 kDa (**cE**), respectively.

synthetic genes under the control of the bacteriophage T7 promoter and places a unique 6×His sequence at the N-terminus of the recombinant protein for purification by immobilized metal affinity chromatography. pET30a-SLPs were transformed into *E. coli* BL21(DE3)pLysS and the encoded proteins were expressed upon IPTG induction. Crude extracts were analysed by polyacrylamide gel electrophoresis and Western Blotting using an anti-His antibody. Staining of the gels with Coomassie Blue showed clear bands of His₆-SLP2, His₆-SLP4, and His₆-SLP6 proteins, which have the molecular weight of 19 kDa, 29 kDa, and 40 kDa, respectively (Figure 3). However, higher order multimers (His₆-SLP8 and His₆-SLP12) which were readily detected by Western Blotting analysis, did not appear as visible bands (data not shown). This has been observed in the expression of other silk-like recombinant proteins.^{25,26} The antibody used in the Western

Figure 4. SDS-PAGE of purified hybrid proteins produced from pET30a-SLP6. **M**: molecular weight marker; **Lane 1**: CNBr-cleaved protein, SLP6; **Lane 2**: uncleaved protein His₆-SLP6.

Blotting assays was not quantitative in its reaction with silk-like proteins but could be reliably used to detect synthetic hybrid proteins both in whole cell lysates and in purified form. In conjunction with the physical characterization of the purified materials (*vide infra*), the data support the expression of SLP multimers using the pET expression system.

Proteins expressed from plasmid pET30a-SLP were purified on a Ni-NTA column through the 6×His sequence positioned at the N-terminus. We focused our characterization efforts on three distinct size variants of the SLP multimer sequences. Multimers containing 2, 4, and 6 repeats of the 38 amino acids, that is, SLP2, SLP4, and SLP6, respectively, were examined. Typical yields for the dried proteins were 21.0, 38.7, and 31.0 mg L⁻¹ of medium culture, respectively, (Table I). These proteins were further purified by CNBr cleavage and then by dialysis. Figure 4 compares the SDS-PAGE results of the His₆-SLP6 (lane 2) and the CNBr-cleaved protein, SLP6 (lane 1). The cleaved product was observed as a single predominant band and uncleaved protein was not remained under the cleavage conditions, suggesting that the purity of this product is high. The molecular weight of the cleaved protein is around 36 kDa. All of the proteins were identified by N-terminal amino acid sequencing (data not shown).

Solid State ¹³C NMR Analysis

The solid-state ¹³C NMR chemical shifts of polypeptides and proteins have been reported to be extremely sensitive to backbone conformation of the constituent residues and have been exploited for the structural analysis of silk fibroin.²⁷ Figure 5 compares the ¹³C CP/MAS solid state NMR spectra of SLP1 (a) and SLP6 (b). As for comparison, the spectra of Cp fraction in silk I and silk II forms are also shown in Figure 5c and 5d, respectively. The Cp fraction predominantly

Figure 5. ^{13}C CP/MAS NMR spectra. **a:** monomeric peptide, SLP1; **b:** hybrid protein, SLP6; **c:** Cp fraction in silk I form; **d:** Cp fraction in silk II form. The conformational assignments of the Ala C $^{\beta}$ peak, I (Silk I) and II (Silk II), are indicated by the dotted lines in the spectra.

Figure 6. Expanded Ala C $^{\beta}$ peaks of ^{13}C CP/MAS NMR spectra of (a) monomeric peptide, SLP1 and (b) hybrid protein, SLP6. The spectral deconvolution was performed by assuming Gaussian.

has the amino acid sequence of $(\text{GAGAGS})_n$.^{9,13} The resonance assignments of the constituent amino acids are identified from their characteristic peak positions and by comparing with reported chemical shift data of the silk fibroins and their derived peptides.²⁷ As judged from the characteristic chemical shift values of the Ala and Gly residues (Ala C $^{\alpha}$ $\sim$ 50.5, Ala C $^{\beta}$ $\sim$ 15.9, Gly C $^{\alpha}$ $\sim$ 42.5 ppm) (Figure 5a), it might be concluded that the SLP1 takes silk I structure. However, the broad line-shape of Ala C $^{\beta}$ peak (Figures 5a and 6a) suggests that it takes a distorted β -turn.^{20,21} On the other hand, Figure 6b shows that the chemical shift of Ala C $^{\beta}$ peak

Figure 7. A comparison of the CD spectra of SLP1 (○) and SLP6 (■) in HFIP solution. The characteristic band positions and the residual molar ellipticity values are described in the text.

of SLP6, as well as SLP2 and SLP4 (data not shown) is significantly different from that of SLP1 although the difference is small. Namely, the presence of small amounts of β -sheet structure is expected in SLP6 sample. The Ala C $^{\beta}$ peak was divided into two peaks after careful peak simulation. The fraction was estimated to be 72% distorted β -turn and 28% β -sheet.^{20,21}

CD Analysis

In order to prepare silk fiber from the hybrid proteins, HFIP was used as the solvent and methanol was used as the coagulant for artificial spinning.^{28,29} However, we could not obtain the fiber. So we tried to characterize the solution structure of SLP1 and SLP6 by the CD method in HFIP solution. The analysis may provide an opportunity to directly correlate the secondary structural features of the silk derived peptides in the specific hydrophobic environment.

Figure 7 compares the CD spectra of SLP1 and SLP6 in HFIP. The observed CD spectrum of SLP1 is characterized by the presence of two weak negative bands: a shoulder at $\sim$ 223 nm ($[\theta]_M \sim -650 \text{ deg. cm}^2 \text{ mol}^{-1} / \text{residue}$) and a relatively sharp band at $\sim$ 202 nm ($[\theta]_M \sim -1680 \text{ deg. cm}^2 \text{ mol}^{-1} / \text{residue}$) and an intense positive extremum at $\sim$ 190 nm ($[\theta]_M \sim -2990 \text{ deg. cm}^2 \text{ mol}^{-1} / \text{residue}$). The crossover exists at $\sim$ 197 nm. Interestingly, these spectral features in case of SLP6 are dramatically pronounced however, without any change in the band positions, *i.e.*, a distinct negative shoulder $\sim$ 223 nm ($[\theta]_M \sim -2720 \text{ deg. cm}^2 \text{ mol}^{-1} / \text{residue}$), an fairly strong band at $\sim$ 204 nm

($[\theta]_M \sim -4950 \text{ deg. cm}^2 \text{ mol}^{-1}/\text{residue}$) and a strong positive peak at $\sim 190 \text{ nm}$ ($[\theta]_M \sim -8800 \text{ deg. cm}^2 \text{ mol}^{-1}/\text{residue}$). While the CD pattern of the monomer unit exhibits a clear inclination for the helical structure, the observed spectra of SLP6 clearly indicate the predominance of helical conformations stabilized by intra-molecularly hydrogen bond. Marked changes in the band intensities of the hexameric unit clearly suggest the existence of cooperative effects in the induction of secondary structures. On the contrary, based on CD studies of elastin-like peptides (VPGVG) $_n$ (where n varies between 1 to 5), the lack of co-operative effects has been suggested.³⁰ Moreover, the 'cooperative unit' did not seem to exceed more than one repeat. Under the present experimental conditions, the results largely exclude the presence of β -sheet structures in solution condition. Of the two well-known, closely related helical secondary structures, *i. e.*, α - and 3_{10} -helices, the observed CD spectra in HFIP appear to be more close to a 3_{10} -helical structure however, the presence of equilibrium between the two helical forms cannot be ruled out. It must be noted that the backbone torsion angles ($\phi \sim -50 \pm 20^\circ$ and $\psi \sim -30 \pm 20^\circ$) characterize both the helical forms and subtle changes in the torsion angles may easily inter-convert the two structures. Interestingly, Toniolo and co-workers indicated the possibility of distinguishing the two helical forms from the experimental CD results from the characteristic ratio $R = [\theta]_{222}/[\theta]_{208}$.^{31,32} For an ideal 3_{10} -helical structures, the characteristic ratio $R = [\theta]_{222}/[\theta]_{204}$, is suggested to be 0.4 whereas, it is close to 1.0 in the case of α -helix. The CD results on the hexameric unit are more in favour of the polypeptides with right-handed 3_{10} -helical conformation, as judged from the relative intensities of the bands at 204 and 222 nm.

CONCLUSIONS

We could not obtain silk fiber from the hybrid proteins with spinning method from the solution; HFIP as the solvent and methanol as the coagulant. However, SLP6 with the longest peptide sequence among the hybrid samples prepared here takes 28% β -sheet structure in the solid state and takes ordered structure like 3_{10} helix in HFIP. Therefore the hybrid silk protein with longer sequence will be possible to prepare the fiber. The present study clearly indicates that combining the two structurally different characteristics from two or more different species may be an attractive possibility for exploring and comparing the functional properties of the silk materials. It appears encouraging that the present protocols for obtaining the hybrid SLPs in sufficient quantity, may further be extended to other related

systems.

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