Folding process of silk fibroin induced by ferric and ferrous ions

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1. Introduction

Silk is a natural protein produced by many insects [1,2]. The silk fiber produced by the silkworm, Bombyx mori, has been used for textiles for thousands of years in China. It has a very useful combination of mechanical properties [3] and is potentially applicable in biotechnological and biomedical materials [4]. The properties are largely determined by the sequence of amino acid and the secondary structure of silk fibroin which is the principle structural protein of silk [5]. Bombyx mori silk fibroin is mainly composed of two subunits: heavy chain (391 kDa) and light chain (28 kDa) [6]. The heavy chain accounts for up to 85 wt.% of the degummed silk [6]. The heavy chain (391 kDa) and light chain (28 kDa) [6]. The heavy chain largely consists of highly repetitive hydrophobic motifs GAGAGS and GA(V)GAGY forming the crystalline regions, while 11 hydrophilic non-crystalline spacers GTGSSFGPYVAN(H)GGYSGYEYAWSSESDFGT are fairly regularly dispersed between the crystalline blocks [6,7]. Silk fibroin is found in mainly two distinct states with different secondary structures: Silk I (helix-form) and Silk II (β-sheet-form) [8–12]. The Silk I state can be converted to the Silk II state by a range of factors including shearing, extensional flow, water removal and heating [3,13]. In addition, acidic pH and metallic ions are also important factors influencing the conformation transition from Silk I to Silk II [14–20]. Our previous investigations have demonstrated that pH value and Cu2+, Ca2+, Zn2+, K+, Na+ ions may influence the folding process of silk fibroin [16–20].

However, there are few reports focused on the role of iron ions in silk fibroin. The content of iron element was 1.8 ± 0.8 μg/g in dope silk while 6.1 ± 0.3 μg/g in cocoon silk detected by atomic adsorption spectroscopy (AAS) [21]. Cocoon silk contains more iron than dope silk, implying iron ions might play a special role in the formation of silk fiber.

In this work, we used solid-state 13C nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR) and Raman spectroscopy to investigate the influence of ferric and ferrous ions on the conformation of silk fibroin and the possible binding sites of ferric or ferrous ions in the silk fibroin. Our results provide further clarification of effects of ferric ions on the conformation transition of silk fibroin in vitro with implications for the role of this ion during the natural spinning process.

2. Experimental

2.1. Sample preparation

Bombyx mori silk cocoons were degummed by boiling in aqueous 0.5 wt.% Na2CO3 solution for 1 h. The degummed silk fibers washed with several changes of ultra-pure water (resistivity ~ 18.2 MΩ cm) and dried at 60 °C without contact with metallic ions. Six grams of degummed silk fibers were dissolved in 100 ml of 9.3 M LiBr solution and the resulting solution was dialyzed...
against ultra-pure water for a total of 3 days to remove LiBr. The ultra-pure water was refreshed every 3 h during dialysis. Finally a 2.5 wt.% regenerated silk fibroin solution was obtained.

A series of regenerated silk fibroin solutions containing different [Fe³⁺] were prepared by mixing 2.5 wt.% regenerated silk fibroin solution and 1.5 × 10⁻⁴ g/ml FeCl₃ solutions together in different proportions. The added contents of ferrous ions relative to the regenerated silk fibroin solutions was 0, 5.0, 10.0, 25.0, 50.0, 75.0, 100.0 and 125.0 μg/g, respectively, and all of these solutions had pH value of 6.8. Cast films (Fe³⁺/SF samples) were prepared for each concentration by allowing 5 ml of mixed solution to dry in a shallow polystyrene weighing boat in a fume hood at room temperature (25 ± 0.5 °C).

For the preparation of Fe²⁺/SF solutions, the ultra-pure water was distilled, cooled and then saturated with nitrogen gas to remove oxygen. The contents of ferrous ions in the regenerated silk fibroin solutions were the same as that of Fe³⁺/SF samples. The prepared Fe²⁺/SF solutions were cast as above but dried in a desiccator containing separate dishes of alkaline pyrogallol and phosphorous pentoxide. All the samples were prepared three separate batches used as replicates.

2.2. ¹³C NMR experiment and spectral simulation

¹³C CP/MAS experiments were performed on Varian UNITY plus-400 NMR spectrometer using the following operating conditions: 100 MHz; contact time of 1.1 ms; pulse repeat time of 5 s long enough for the longitudinal relaxation of the ¹³C spin nucleus [22]; accumulation over 1000 scans; magic angle spinning rotor of levels 1 to 6 in proper axial orientation, while the zero-field splitting (ZFS) interaction is either zero or 1/3, indicating an axial or fully rhombic symmetric system, respectively. In addition to the degeneracy levels lifted by ZFS, the remaining degeneracies are further lifted to the six non-degeneracy levels in the applied magnetic field. In a high magnetic field (gβB ≫ D), the six levels correspond to pure Ms = ±1/2, ±3/2, ±5/2 spin states and only transitions following the selection rule ΔMs = ±1 are allowed. However, as ferric ions exist at a high-spin state (S = 5/2) and a purely rhombic symmetry (E/D = 1/3) under the low magnetic field (gβB < D), the transitions no longer follow the selection rule because of the contribution of second and third terms in Eq. (1). The resonance transitions occur between each of pair levels 1 → 2, 3 → 4, 5 → 6 where the energy increases in turn from level 1 to level 6 [33]. The pair of levels for each Kramer doublet can be treated as a fictitious spin S = 1/2 with an effective or apparent g-value of g' determined by the field position of the resonant absorption in the EPR spectrum of Fe³⁺/SF samples. The weak resonance at g' ~ 9.7 and 0.6 in the spectrum are derived from the transitions of levels 1 → 2 and 5 → 6 in proper axial orientation, while the strong isotropic resonance at g' ~ 4.3 is derived from the transition of levels 3 → 4 [33]. The situation with g' ~ 4.3 is most frequently observed in non-heme iron atoms.

In this work, EPR spectra were recorded on a Bruker EMX-8/2.7 spectrometer using the following parameters: specimen temperature 100 K; microwave power 2.003 mW at a frequency of 9.45 GHz; modulation amplitude of 4 G and frequency of 100 kHz; Sweep width 4000 G. Diphenylpicrylhydrazyl (DPPH), g = 2.0036 was used as an external reference. The simulation of EPR spectra was carried out using Easyspin 2.7 software supplied by Swiss Federal Institute of Technology (URL: http://www.easyspin.org/).

The EPR spectrum of Fe³⁺/SF samples was not detectable under the experimental conditions available to us.

3. Results

3.1. Conformational dependence of silk fibroin on ferric or ferrous ions

Fe³⁺/SF and Fe²⁺/SF samples were studied with ¹³C NMR spectroscopy to compare the different effects of ferric and ferrous ions on the conformation of silk fibroin. Fig. 1A is an example to show the full scale ¹³C NMR spectrum of Fe³⁺/SF sample with added
only increased slightly (Fig. 1C-b). It suggested that ferric and ferrous ions with low concentrations could stabilize Silk I structure while at concentrations more than 75 μg/g ferric ions markedly promote the conformation transition from Silk I to Silk II in a dose-dependent fashion while ferrous ions have only a small promoting effect.

3.2. Effect of ferric or ferrous ions on the $I_{854/830}$ ratio in Raman spectra of SF

The phenolic moiety of tyrosine residue exhibits intense Raman doublet resonance in the region of 820–860 cm\(^{-1}\). The doublet resonances at 854 and 830 cm\(^{-1}\) and their ratio, $I_{854/830}$ are used as an indicator of the strength of the hydrogen bonding to/from the phenoxyl-OH group which often forms a hydrogen bond in certain environments [28]. In general, the ratio of $I_{854/830}$ in globular proteins lies between 0.3 and 2.5. When the tyrosine phenoxyl proton is a strong hydrogen-bond donor, $I_{854/830} = 0.3$; while when the tyrosine phenoxyl oxygen is a strong hydrogen-bond acceptor, $I_{854/830} = 2.5$ [28]. However, for the non-hydrogen-bonded phenoxyl group often seen in non-globular proteins, the intensity around 830 cm\(^{-1}\) goes down while the intensity of 854 cm\(^{-1}\) is very high. As a result, the ratio of $I_{854/830}$ exceeds 2.5 indicating a hydrophobic microenvironment for the tyrosine residues [34–36]. Tyrosine is comparatively abundant in silk fibroin heavy chain [6] accounting for about 5% of the amino acid residues and can be taken as a sensitive probe to detect changes of microenvironment around Tyr-containing domains.

Fig. 2A shows a Raman spectrum for a Fe\(^{3+}\)/SF sample with added [Fe\(^{3+}\)] of 75 μg/g. The peaks at 830 and 854 cm\(^{-1}\) are marked with single and double asterisks, respectively. Fig. 2B shows de-convolution of peaks in range of 895–810 cm\(^{-1}\). Fig. 2C-a and C-b, respectively, show the effect of [Fe\(^{3+}\)] and [Fe\(^{2+}\)] on the $I_{854/830}$ ratio in Raman spectra of SF samples. In the absence of ferric or ferrous ions the $I_{854/830}$ ratio is about 3.25 but changes as [Fe\(^{3+}\)] or [Fe\(^{2+}\)] is increased. The trend of $I_{854/830}$ ratio in Fe\(^{2+}\)/SF samples (Fig. 2C-b) is different from that in Fe\(^{3+}\)/SF samples (Fig. 2C-a). $I_{854/830}$ ratio first increased rapidly when [Fe\(^{2+}\)] was lower than 5 μg/g, but plateaued when the [Fe\(^{2+}\)] reaches about 75 μg/g (Fig. 2C-b). In contrast, the effect of ferric ions on the $I_{854/830}$ ratio is more complicated. When [Fe\(^{3+}\)] was lower than 10 μg/g, it appeared to have little effect, the $I_{854/830}$ ratio remaining about 3.2 indicating a moderately hydrophobic environment. Thereafter for [Fe\(^{3+}\)] between 25 and 75 μg/g, the $I_{854/830}$ ratio decreased considerably to about 2.3, which indicated a more hydrophilic environment in which the phenoxy group acted as a proton acceptor. Increasing the [Fe\(^{3+}\)] from 75 to 125 μg/g raised $I_{854/830}$ to about 3.3 (Fig. 2C-a), which indicated a more hydrophilic Tyr environment.

3.3. EPR spectra of Fe\(^{2+}\)/SF samples

Fe\(^{2+}\)/SF samples with [Fe\(^{2+}\)] of 75.0 and 125.0 μg/g were measured by EPR spectrometer. Fe\(^{3+}\)/SF sample with [Fe\(^{3+}\)] of 75.0 μg/g has the lowest Silk II content while that with [Fe\(^{3+}\)] of 125.0 μg/g has the highest Silk II content, but they have a similar EPR spectrum as shown in Fig. 3a with [Fe\(^{3+}\)] of 75.0 μg/g. Only one signal at $g \approx 4.25$ was observed, which implied that the transition occurred between energy levels 3 $\rightarrow$ 4 [33]. The EPR signal of pure FeCl\(_3\) sample was not observed (data not shown). Fig. 3b is the simulated spectrum. The parameters extracted from the simulation are as follows: the zero-field-splitting interaction $D = 1.2$–2 cm\(^{-1}\) which is larger than the applied magnetic field $B$ of 0.315 cm\(^{-1}\) (i.e. X-band) in our experiment; $E/D = 1/3$; the theoretic $g$-value $= 1.950$, 1.990, 1.995. Based on the apparent $g$-value, $g = 4.25$. 

Fig. 1A shows a 13C NMR spectrum of a Fe\(^3+/SF\) sample with added [Fe\(^{3+}\)] of 75.0 μg/g and the peak for alanine C\(_b\) is marked with asterisk; (B) the simulation of alanine C\(_b\) peak in 13C NMR spectrum with four structural components for the same sample: (a) helix-like (15.0 ± 0.5 ppm), (b) helix (17.0 ± 0.5 ppm), (c) β-sheet (20.0 ± 0.5 ppm), (d) β-sheet-like (21.5 ± 0.5 ppm); (C) the dependence of Silk II contents (including β-sheet and β-sheet-like components) on added [Fe\(^{3+}\)] in silk fibroin: (a) Fe\(^{3+}\)/SF samples, (b) Fe\(^{2+}\)/SF samples. The simulation error is ±2%.

[Fe\(^{3+}\)] of 75.0 μg/g, and the peak of alanine C\(_b\) is marked with asterisk. Fig. 1B indicates the de-convolution of alanine C\(_b\) peak with four structural components, wherein (a–d) represents, respectively, the components of helix-like (15.0 ± 0.5 ppm), helix (17.0 ± 0.5 ppm), β-sheet (20.0 ± 0.5 ppm) and β-sheet-like (21.5 ± 0.5 ppm) [11]. The effect of the ferric and ferrous ions on the Silk II contents is shown in Fig. 1C. Within the range 0–75.0 μg/g of iron ions contents, Silk II contents for Fe\(^{2+}\)/SF and Fe\(^{3+}\)/SF are closely comparable: slowly decreasing but lying between 17% and 22%. However, when iron ions exceed 75 μg/g, Silk II content of Fe\(^{2+}\)/SF samples increased progressively and markedly to about 40% at 125 μg/g (Fig. 1C-a), but that of Fe\(^{2+}\)/SF samples
and those simulated parameters, we could conclude that the ferric ions in the silk fibroin are at high-spin state of $S = 5/2$ and low symmetric site [37].

4. Discussion

4.1. Coordination sites of ferric ions in silk fibroin

Bou-Abdallah and Chasteen [33] assigned the EPR signal of $g' = 4.25$ with zero-field-splitting interaction $|D| \leq 2 \text{ cm}^{-1}$ and $E/D = 1/3$, to a mononuclear high-spin ferric ions ($S = 5/2$) in a site of low symmetry, which is often observed in many proteins. These proteins include non-heme iron proteins [38], for example, transferrin [39], protocatecuate dioxygenase [40], bacterial iron carrier proteins [41] as well as various siderophores [42]. Our Fe$^{3+}$/SF sample has a very similar EPR signature to those proteins.

Tyrosine, histidine, glutamine and aspartate generally have a strong ability to coordinate with ferric ions [43]. In the Fe$^{3+}$-transferrin, it is believed that each chelating site involves two imidazole groups and three tyrosyl residues [44]. In human transferrin N-lobe, the ferric ions binds to Asp63, Tyr95, Tyr188 and His249 residues and two oxygen atoms from the bidentate carbonate ion [45]. In lipoxygenases of human [46], rabbit [47] and soybean [48], the binding sites of ferric ions include His, Asn and Ile residues. All of these residues are located in link regions of the proteins. Interestingly, the 11 highly conserved hydrophilic spacers TGSSGFGPY-VAN(H)GGSGYEYAWSSSDFGT in the heavy chain of silk fibroin also play a role as a linker or spacer and covers all of residues which are considered as potential binding sites for ferric ions (Fig. 4). The spacer in silk fibroin connects two regularly arranged sequences, i.e. (GAGAGS)$_n$ and/or (GA(V)GAGY)$_n$ in helix-form (Silk I) or β-sheet form (Silk II). As a result, silk fibroin might bind to ferric ions with those residues in the hydrophilic spacers. If ferric ions

Fig. 2. (A) Raman spectrum of Fe$^{3+}$/SF sample with added [Fe$^{3+}$] of 75.0 µg/g and peaks at 830 cm$^{-1}$ and 854 cm$^{-1}$ are marked with asterisk and double asterisks, respectively; (B) the de-convolution of peaks in Raman spectrum within interval of 895–810 cm$^{-1}$ for the same sample; (C) the dependence of $I_{854}/I_{830}$ ratio on iron element content for Fe$^{3+}$/SF samples (a) and Fe$^{2+}$/SF samples (b). The error was resulted from the different batches of experiments.

Fig. 3. The experimental (a) and simulated (b) EPR spectrum of Fe$^{3+}$/SF sample with added [Fe$^{3+}$] of 75.0 µg/g under the magnetic field strength of 9.45 GHz, $T = 100$ K. The simulated parameters are as follows: $g_1 = 1.950$, $g_2 = 1.990$, $g_3 = 1.995$, $D = 2 \text{ cm}^{-1}$ and $E/D = 1/3$ and the line width peak to peak is 4 MHz.

and those simulated parameters, we could conclude that the ferric ions in the silk fibroin are at high-spin state of $S = 5/2$ and low symmetric site [37].
were trapped in such regions, the β-sheet folding center might be formed, and thereafter the folding process could be accelerated if more ferric ions were added [49].

4.2. Influence of ferric ions on secondary structure of silk fibroin

NMR results show that the Silk II content of silk fibroin does not increase considerably until [Fe³⁺] is as high as 75.0 μg/g (Fig. 1C). Moreover, the top x-axial in Fig. 1C shows the molar ratio of [Fe³⁺]/[spacer] of heavy chain silk fibroin. Note that until the ratio of [Fe³⁺]/[ spacer] reaches 1:1, the content of Silk II increases markedly. It indicated that a small amount of ferric ions could maintain the ratio of [helix-form]/[β-sheet form] as constant in silk fibroin. But if more ferric ions were added, more β-sheet structures would be formed due to the interaction of ferric ions with the specific residues in spacers. Once the folding template was formed, the folding process would be marked accelerated [47] because of the strong hydrophobic interactions between hydrophobic spacers in the silk fibroin, leading to the aggregation of β-sheet components. The process was demonstrated to be nucleation dependent [50].

4.3. Fe³⁺-induced correlation between secondary structure and Tyr-related domain environment in silk fibroin

NMR and Raman spectra can provide complementary information about the secondary structure and Tyr-related domain environment in the silk fibroin. The I₈₅₀/I₈₃₀ ratio in Raman spectrum reflects the microenvironment of tyrosine-related domains, whereas NMR spectra yield information on secondary structure. Most of the tyrosine residues are located in the repeated peptides (GA/VGAGY)ₙ of silk fibroin. Taddei et al. used Raman spectroscopy to investigate the semi-crystalline domains with series of (AG)ₙr-based peptides containing tyrosine, valine and serine, such as (AG)ₙ₁₋₅, (AG)₉Y(AG)₂, (AG)₉₂Y(AG)₂, etc. [51]. They found that the I₈₅₀/I₈₃₀ ratio for the typical Silk I Cp (chymotryptic precipitate fraction) is as high as 5.6, indicating a rather hydrophobic microenvironment around the tyrosine residues (Y). The I₈₅₀/I₈₃₀ ratio for the typical Silk II Cp is 2.9 much lower than that for the Silk I Cp, but it is still higher than 2.5, which is the upper limit for globular proteins [28]. The I₈₅₀/I₈₃₀ ratio goes down with the increase of tyrosine residues in the peptides. Also, they noted that the location of tyrosine either in the end or in the middle of peptide chain also influences the value of I₈₅₀/I₈₃₀ [51]. Fig. 2C-a shows that when [Fe³⁺] is below 10.0 μg/g, the I₈₅₀/I₈₃₀ ratio is about 3.25, implying a hydrophobic microenvironment around the tyrosine residues. Meanwhile, as shown in Fig. 1C, the helix-form dominates structure of silk fibroin. However, when [Fe³⁺] is within 20–75 μg/g, the I₈₅₀/I₈₃₀ ratio drops to around 2.3. During this process, the environment of Tyr-related domain becomes moderately hydrophobic. The secondary structures of silk fibroin might be adjusted to a loose intermediate, i.e. helix-like and/or β-sheet-like forms. When [Fe³⁺] is higher than 100.0 μg/g, the I₈₅₀/I₈₃₀ ratio returns to 3.3, indicating a hydrophobic microenvironment around the tyrosine residues since considerable amount of Silk II structures are formed (Fig. 1C).

The folding mechanism of large proteins is complicated [52] because these proteins are constructed of foldons that can separately fold through nucleation-condensation and then dock. Nucleation and docking events depend on the stability of individual foldons and how the nucleation and docking are coupled. Under most conditions, the formation of one foldon is a rate-determining process that appears a transition state in which one foldon alone is partially formed and the other foldons would not form until the high energy intermediate state is present. The folding process would be speeded up by the cooperative effects [53]. Li et al. [50] demonstrated that the fibrosis process of silk fibroin submits to a nucleation-dependent mechanism. During the process, ferric ions might act as a folding core, leading the inter-molecular interaction of two adjacent hydrophobic domains.

Based on previous discussion and the microstructure model of silk fiber proposed by Ha and Asakura [54,55], we suggest that ferric ions induce a three-states folding mechanism of silk fibroin. In State I, there are few ferric ions present. The silk fibroin conformation is dominated by helix-form, where the phenoxyl groups of Tyr residues tend to form H-bond with adjacent chains. In State II, more ferric ions are present. The ferric ions locate in the spacer domain, facilitating adjustments of hydrophobic domain to form helix-like and β-sheet-like structures and even form some β-sheet foldons. At this state the two adjacent hydrophobic chains are still far away so that the phenoxyl group of Tyr is difficult to reach the adjacent chain. H₂O molecules fill into the gap between two adjacent chains, leading to a less hydrophobic environment around the tyrosine [54,55]. In State III, more ferric ions appear, leading to the formation and aggregation of more β-sheet structures. In such a situation, the phenoxyl groups of Tyr residues in two adjacent chains may interact in a parallel orientation to lower the system energy, and therefore results in a hydrophobic environment around the Tyr residues. The folding process of silk fibroin resembles that of fibrosis proteins like Aβ42, α-synuclein, prion, etc., which are relevant to neurodegenerative diseases.

4.4. Role of ferrous ions in silk fibroin

¹³C NMR results indicate that increasing [Fe²⁺] does not considerably increase the Silk II content. The I₈₅₀/I₈₃₀ ratio in Raman spectra stabilizes at 4.25 when [Fe²⁺] exceeds 10.0 μg/g, and this ratio is comparable with that for the Silk I Cp, 5.6. Therefore, it seems ferrous ions have no obvious effects on the folding process of silk fibroin, and Silk I is the dominant structure of silk fibroin with [Fe²⁺] of 10.0–125.0 μg/g. Recently, EfuU (Escherichia coli Fe Uptake) protein, a Fe²⁺-uptake transporter from E. coli, is regarded as a possible Fe²⁺-permease in proteoliposomes in vitro [56]. Topological analysis indicates that EfuU is an integral cytoplasmic mem-

![Fig. 4. The repetitive sequence of spacer in silk fibroin heavy chain. The potential binding sites are marked with colors, including H (histidine), Y (tyrosine), N (asparagine), E (glutamic acid) and D (aspartic acid).](image-url)
brane protein, exhibiting seven transmembrane helices. Two REXXE motifs in the transmembrane helices of OfeT (oxidase-dependent iron transporters) were thought to bear the ferrous ions translocation [56]. Interestingly, there exists a similar motif REGYE in heavy chain of silk fibroin. The motif REGYE locates in the C-terminal of silk fibroin, influencing the integrated structure of silk fibroin to a much lower extent [6]. Consequently, if ferrous ions located in this domain, it would hardly make conformational changes of silk fibroin.

As for the reason why ferric and ferrous ions have different effects on the conformation of silk fibroin, we consider it is possibly stemmed from the different electrostatic interaction of two types of ions with silk fibroin. The ferric ions have higher positive charge than ferrous ions, resulting in a stronger interaction with negative charge residues in silk fibroin.

5. Conclusion

Ferric ions with concentration exceeding 125.0 μg/g can dramatically change the regenerative silk fibroin conformation from Silk I to Silk II. The contents, however, are much higher than those naturally occurring in silkworm glands, but are necessary due to the absence of effective cooperative factors including shearing stress, extensional flow, condensation, etc. Tyr-related domains in hydrophobic environments, if induced by more ferric ions, will facilitate the formation of Silk II. Ferric ions in silk fibroin have high-spin states (S=5/2) with low symmetry and might bind to His, Tyr, Ile and Asn residues located in the spacer regions of silk fibroin. Ferric ions with low concentration could stabilize silk fibroin in helix structure but high concentration of ferric ions would induce the formation of β-sheet structure in silk fibroin. Different from ferric ions, ferrous ions have less influence on the conformation of silk fibroin. Ferrous ions might bind the residues in motif of REGYE lying in the C-terminal of silk fibroin. Moreover, these results seem also meaningful to understand proteins like Aβ-synuclein. These proteins are considered relevant to Alzheimer's disease and Parkinson's disease, where iron elements deposit in the infected brain tissues [57,58], and the protein structure is transformed from a highly soluble conformation to an insoluble β-sheet rich structure [59].

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