Copper in the silk formation process of *Bombyx mori* silkworm

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Abstract Evidence is presented here that cupric ions play a part in the natural spinning of *Bombyx mori* silk. Proton induced X-ray emission studies revealed that the copper content increased from the posterior part to the anterior part of silk gland, and then further increased in the silk fiber. Spectrophotometric analysis demonstrated that cupric ions formed coordination complexes with silk fibroin chains while Raman spectroscopy indicated that they induced a conformation transition from random coil/helix to β-sheet. Taken together these findings indicate that copper could play a role in the natural spinning process in silkworms.

Key words: Copper content; Silk spinning; Complex formation; Conformation transition; Spectrophotometry; *Bombyx mori* silk fibroin

1. Introduction

Natural silk has a long history of use as a textile fiber, and has been used in recent years for medical sutures blood vessels, artificial skin and tendons, etc. [1]. The remarkable tensile and thermal properties of silk fibers, especially those of spider draglines, have attracted considerable interest in fundamental research and potential industrial application [2,3]. Partial and complete gene sequences have been reported for the major structural proteins in silks from a range of lepidopteran species and for a range of different silk types in a range of different spider species [4]. Interest has also focused recently on the nanofibrillar construction of silks [5] and on natural silk processing [3]. Though several different methods have been tried for spinning silk proteins artificially, the mechanical properties of the resulting fibers have proved much worse than the natural ones [6–10]. Moreover, artificial spinning conditions involve extreme processing conditions, such as high pressure and toxic solvents in sharp contrast to the mild processing conditions in vivo, which include aqueous medium, ambient temperature, low hydrostatic pressure, and low extrusion rates and draw down ratios [11].

There is good evidence that the excellent properties of silk fibers depend as much (or more) on the spinning process as (than) on the protein sequence and structure [12,13]. It is widely accepted that the spinning process involves a conformation transition in the silk protein (fibroin or spidroin) from random coil/helical conformations to β-sheet conformation produced by shearing and/or rapid extensional flow converting a highly aqueous concentrated solution of silk protein into an insoluble solid. In addition, a number of studies showed that other factors, such as metallic ions and pH may be involved in natural silk processing in silkworms and spiders [14–18]. These revealed successive changes in pH and the contents of the metallic ions (for example, Na^+, K^+, and Ca^{2+}) as silk proteins flow through the secretory pathway. pH and metallic ions have profound effects on the rheology of silk protein solutions [17,18] suggesting that these factors may play an important role in the natural silk spinning process. Nemoto and co-workers [18] suggested that calcium and magnesium ions may be important in silkworm silk formation, while potassium ions are thought to be important in spider dragline silk [16,17,19].

In this article we have focused on copper. We present evidence that the copper concentration increases in the latter part of the secretory pathway. We also present evidence that cupric ions interact with silk fibroin and can initiate the conformation transition seen in this protein.

2. Materials and methods

2.1. Materials

Whole silk glands were removed from fifth instar *Bombyx mori* silkworms approximately 12 h before spinning. The epithelium was not removed from the gland to reduce the risk of loss of metallic ions from the luminal contents. The gland was briefly rinsed with de-ionized water to remove hemeolymph, and then blotted with tissue paper. Some glands were cut into three parts: part A, the posterior division plus posterior part of middle division; part B, the middle part of middle division; and part C, the anterior division plus anterior part of middle division [20]. All glands or parts of glands were dried in an oven at 80°C to constant weight. Two types of silk fiber were also examined without further treatment: (i) silk directly reeled from silkworm spigot; (ii) silk cut from the outer part of a naturally dried cocoon. The dilute regenerated silk fibroin aqueous solution [2.0% (w/w)] was prepared by dissolving degummed silk in aqueous 9.3 mol l^{-1} LiBr solution, and then dialyzing it against de-ionized water for 3 days at 20°C. Details of the procedure can be found elsewhere [21]. The method for obtaining concentrated silk fibroin aqueous solution [26.0% (w/w)] is the subject of a Chinese patent application (application no. 03142201.2).

2.2. Proton induced X-ray emission (PIXE) measurements

PIXE was used to determine the copper content in both silk glands and silk fibers. The experimental conditions were as previously described [22]. All observations were repeated three to six times and averaged.

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Abbreviations: PIXE, proton induced X-ray emission; PrP, prion protein; NR, neutral red

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2.3. Raman spectroscopy

Spectra were recorded with a Dilor LabRam-1B Raman microscope from silk fibroin solutions. A He–Ne laser was used to give 4.7 mW of energy at 632.81 nm.

2.4. Spectrophotometry

Spectrophotometry is a highly sensitive method widely used in analytic chemistry [23,24] to detect trace concentrations of transition elements and has been widely used in amino acid and protein research, for example, for the detection of the complex formation of Cu(II) and histidine [25,26]. The neutral red (NR) fading method is one of the applications of UV spectrophotometry to determine copper content [27], and here we used it to study the interaction of cupric ions with silk fibroin solutions. Twenty-five ml of a reagent containing 3 ml 0.1 mol l^{-1} H_2SO_4, 2 ml 1.5% (w/v) H_2O_2, 5 ml 0.0005% (w/v) NR and different concentrations of cupric ions ranging from a final concentration of 0.05 to 0.5 μmol l^{-1} was freshly prepared and mixed with different volumes of 2% (w/w) silk fibroin solution (the range of final silk fibroin concentration was from 0.04 to 400 mg l^{-1}). The mixtures were heated in boiling water for 15 min, and then cooled immediately under a tap before determining absorbance at 521.4 nm (optical path length of 1.0 cm) at least three times for each cupric concentration using a Lambda 35 UV-Visible spectrophotometer interfaced to a PC computer.

3. Results and discussion

3.1. Determination of copper content of B. mori silk glands and silk fibers

There are some reports concerning changes in metallic ion content in different parts of the silk secretory pathway. Magoshi and Nemoto found that the calcium content was higher in the cocoon wall of *B. mori* than in its silk gland [15] and suggested that it may be involved in the aggregation of silk fibroin [18]. In the spider *Nephila*, energy dispersive X-ray analysis of rapidly frozen hydrated specimens suggested that the potassium ion concentration increased along the duct of the major ampullate silk gland [14] while addition of these ions to dilute solutions of native silk protein (spidroin) encouraged a structural transition leading to β-sheet formation [16,17].

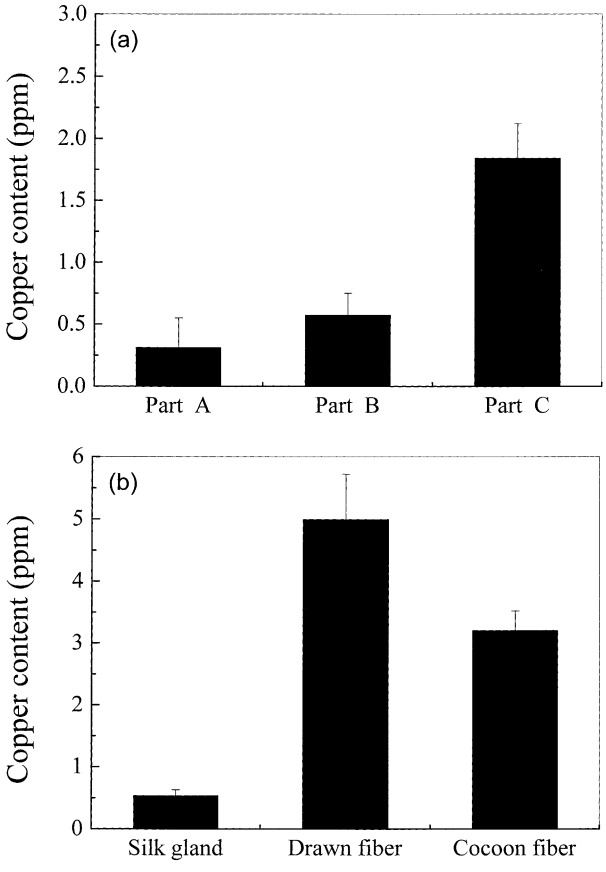

Fig. 1. Copper content determined by PIXE. a: Different parts of the silk gland. b: A comparison between the dried gland and silk fibers of *B. mori* silkworm.

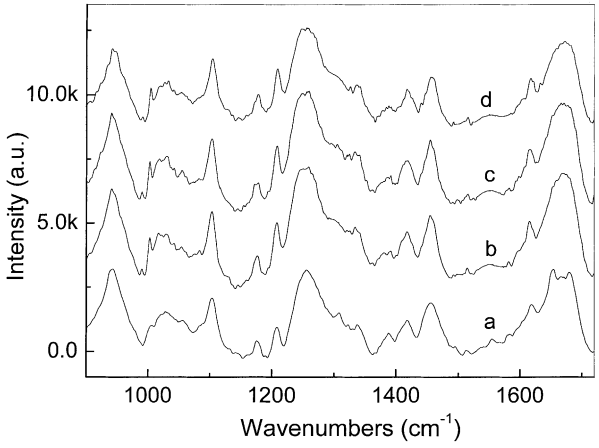

Fig. 2. Raman spectra of silk fibroin solution with different $CuSO_4$ concentrations. CuSO4 concentrations: (a) 0, (b) 0.02, (c) 0.1, and (d) 0.2 mol l^{-1}.

In the present paper we focus on cupric ions. PIXE results clearly demonstrated that the copper content increased from the posterior part to the anterior part of the silk gland, and further increased in the silk fiber. Fig. 1a shows how the copper content increased from posterior to anterior in the silk gland. The copper content appeared to increase progressively from part A (0.31 ± 0.24 ppm) to part C (1.84 ± 0.28 ppm). The average copper content of the whole silk gland (0.53 ± 0.10 ppm) was close to that of part B (0.57 ± 0.18 ppm). The copper content in the silk fiber was much higher than in the gland (Fig. 1b). Although there was some difference between the force drawn fiber pulled from the living silkworm (4.99 ± 0.73 ppm) and the cocoon (3.20 ± 0.30 ppm), both were almost an order of magnitude larger than the average value of the whole silk gland.

3.2. Conformation transition of silk fibroin in the presence of cupric ions

In order to investigate the effect of copper on the conformation transition of silk fibroin, we dialyzed concentrated silk fibroin aqueous solution [26.0% (w/w)] against excess aqueous 0.01 to 0.2 mol l^{-1} $CuSO_4$. The concentration of silk fibroin solution used here was similar to the concentration in silk gland [18] but the copper ion concentrations were at least 1000 greater than those estimated for the fiber by PIXE. The higher copper concentrations were chosen to increase the chance of obtaining a detectable effect in a reasonable length of time.

It is well known that Raman spectroscopy is a useful tool to determine the conformation of silk protein. The silk protein displayed the characteristic conformational sensitive bands in the range 1650–1667 cm^{-1} assigned to amide I, and in the range 1279–1241 cm^{-1} assigned to amide III [28–32]. Fig. 2

shows the effect of different concentrations of $CuSO_4$ solution on Raman spectra of silk fibroin solution. The neat silk fibroin solution (curve a) showed the main characteristic absorption at 1652 cm^{-1} (amide I) and 1259 cm^{-1} (amide III) assigned to random coil/helical conformations. In addition, we found a shoulder peak at 1682 cm^{-1} in the amide I region. The same phenomenon has also been observed in P22 scaffolding protein and was assigned to an 'extended conformation' [33]. We therefore, suggest that the band at 1682 cm^{-1} might be an intermediate conformation between random coil/helix and β-sheet as suggested on the basis of ^{13}C solid state nuclear magnetic resonance [34]. When a low concentration of cupric ions was introduced into silk fibroin solution (curve b, 0.02 mol l^{-1} $CuSO_4$), the center of amide I adsorption band shifted to 1671 cm^{-1} characteristic peak of β-sheet, and the adsorption peak at both 1652 and 1682 cm^{-1} almost entirely disappeared. Moreover, an adsorption peak at 1245 cm^{-1} developed confirming the formation of β-sheet. However, a further increase of Cu(II) concentration did not make much difference in Raman spectra (curves c and d) other than an increase of adsorption intensity at 1245 cm^{-1} in the amide III region.

Thus fairly dilute solutions of $CuSO_4$ can induce the conformation transition in aqueous solutions of silk fibroin from random coil/helical conformation to β-sheet. This is of considerable interest in that Cu(II) is known to induce the formation of β-sheet structure in other proteins [35,36]. Of these the prion protein (PrP) is most instructive. Several studies reveal that cupric ions and altered pH can initiate a conformational transition from α-helix-rich to β-sheet-rich structure in the cellular isoform of PrP (PrPc) [37–39]. In addition, circular dichroism [39–42], electronic paramagnet resonance [39–43], Raman spectroscopy [44], mass spectrometry [45] and X-ray absorption fine structure [46] have been used to demonstrate that Cu(II) can induce the molecular aggregation of PrP by binding to the prion octapeptide. A comparison of the effects of cupric ions on PrP and fibroin further emphasize the similarity [47] between these proteins.

3.3. *Spectrophotometric analysis of the interaction of copper and silk fibroin macromolecules*

Cupric ions catalyze the fading reaction of NR enabling the reaction to be used to detect trace concentrations of these ions. This reaction can be written as follows:

$$\mathrm{NR} + \mathrm{H_2O_2} \xrightarrow[\mathrm{H^+}]{\mathrm{Cu^{2+}}} \mathrm{Oxide}$$

If the concentration of H_2O_2 in the reaction system is much larger than NR, the reaction can be considered as a first-order reaction described by the following equations:

$$\frac{\mathrm{d}C_t}{\mathrm{d}t} = K(C_0 - C_t)C_{\mathrm{Cu(II)}} \quad (1)$$

$$\log\frac{C_0}{C_0 - C_t} = KC_{\mathrm{Cu(II)}}t \quad (2)$$

where C_0 is the initial NR concentration and C_t the oxide concentration at time t. According to Bill's Law, Eq. 2 can be rewritten as follows:

$$\log\frac{C_0}{C_0 - C_t} = \log\frac{A_0}{A_t} = KC_{\mathrm{Cu(II)}}t \quad (3)$$

where A_0 and A_t are the absorbance of NR at the start and at time t, respectively. According to Eq. 3, $\log(A_0/A_t)$ should be directly proportional to the Cu(II) concentration if the reaction time t is kept constant [In this article, the reaction time was set at 15 min, so we used $\log(A_0/A)$ instead of $\log(A_0/A_t)$ in the later discussion].

Fig. 3 indicates that $\log(A_0/A)$ increased with Cu(II) concentration as predicted by the equation. The fading reaction was obviously hindered by the addition of silk fibroin. By comparing the slope of two curves in Fig. 3, we found only 10% of NR was oxidized in the solution containing 40 mg l^{-1} silk fibroin, indicating that most of Cu(II) ions lost their catalytic activity. These observations strongly suggest that Cu(II) bind tightly to silk fibroin in solution.

Although the site of binding of Cu(II) to fibroin is unknown, these ions have a high affinity for the C-terminal and N-terminal region of PrP, and the multi-octarepeat peptide (HGGGWGQ) in the N-terminal region appears to be particularly important [37–46]. Though there are different models for the binding of Cu(II) to particular atoms of PrP, such as [2N, 2O] [39], [3N, 1O] [41–44], [2N, 1O, 1S] [48], the common view is that the N atom of the histidine imidazole

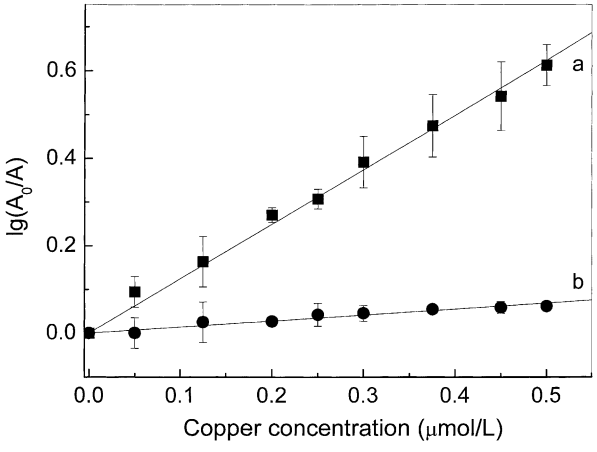

Fig. 3. The relationship between $\log(A_0/A)$ and Cu(II) concentration in copper catalyzed NR fading reaction. a: Control, containing no added protein; b: with 40 mg l^{-1} silk fibroin.

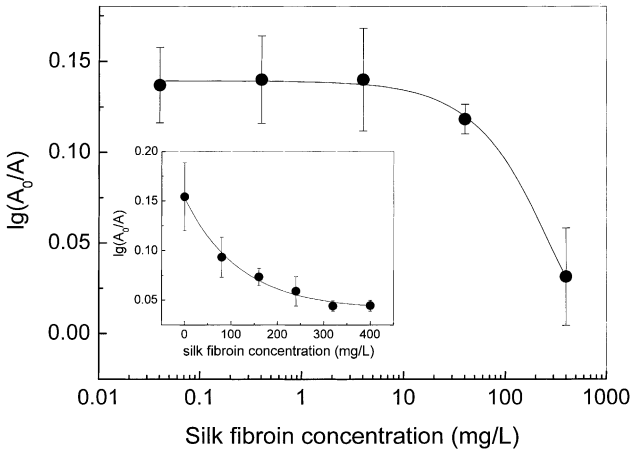

Fig. 4. The relationship between $\log(A_0/A)$ and silk fibroin concentration in copper catalyzed NR fading reaction. Copper concentration: 16 μg l^{-1}.

and the N, O atoms of glycine on the peptide backbone supply ample binding sites for Cu(II). Generally, the imidazole of histidine is thought to be fairly important to the high affinity of PrP for Cu(II), and the histidine on different protein chains can form N2–Cu(II)–N2 intermolecular interaction resulting in molecular aggregation [44]. Moreover, Stöckel et al. have presented evidence that the binding of Cu(II) also promotes the conformational transition of the protein from a predominantly α-helix to a β-sheet structure [39]. This is intriguing in that we have suggested that the protonation of histidine is important for the pH induced aggregation and β-sheet transition in spider silk [17].

It is known that silk fibroin contains various amino acid residues with free $–NH_2$ group or –OH groups, such as histidine, lysine, serine, and tyrosine, so it is reasonable to assume that Cu(II) could form a complex with fibroin macromolecules. As in PrP, the histidine and glycine residues present in silk fibroin could also bind Cu(II).

Further experiments with NR were used to study the binding of Cu(II) to silk fibroin. Fig. 4 demonstrates the effect of silk fibroin concentration on the catalytic activity of copper. Here we kept the Cu(II) concentration at 16 μg l^{-1} (∼0.25 μmol l^{-1}). In one series, the weight ratios of silk fibroin to copper ranged from 2.5 to 25 000, changing an order of magnitude at each interval; and in another series it was increased from 2500 to 25 000 linearly. When the ratio was no more than 250, $\log(A_0/A)$ was practically constant around 0.14, almost the same (ca. 0.15) when silk fibroin was omitted from the reaction system. This meant most of Cu(II) was free when silk fibroin/Cu(II) ratios were lower than 250. However, when the ratio was larger than 250, the $\log(A_0/A)$ value began to decrease. When the ratio increased to 25 000, the $\log(A_0/A)$ value decreased dramatically indicating a marked increase in complex formation and catalytic activity hindrance. Fig. 4 shows how $\log(A_0/A)$ value gradually decreased with the increase of silk fibroin/Cu(II) ratio from 2500 to 25 000. This indicates that only a small number of residues per fibroin molecule bind Cu(II) and only when these are saturated will catalytic activity increase with the addition of more cupric ions. Thus copper may bind to histidine or lysine, which constitutes only 0.2% and 0.3% [49] of the amino acid composition of silk fibroin.

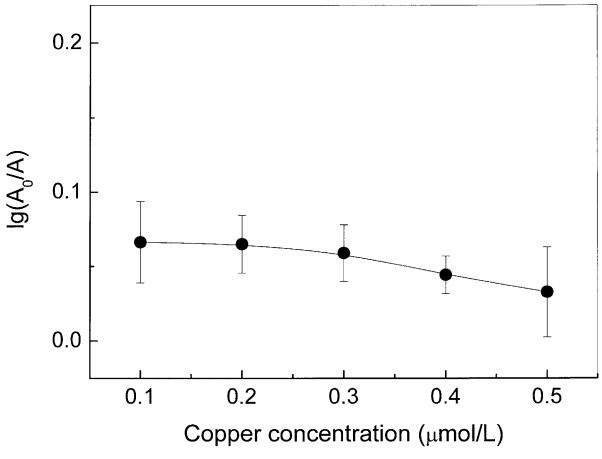

Fig. 5. The effect of Cu(II) concentration on $\log(A_0/A)$ in the copper catalyzed NR fading reaction in the presence of silk fibroin. The weight ratio of silk fibroin to copper was 25 000: 1.

Further confirmation of our assumptions was obtained by keeping the ratio of silk protein/Cu(II) constant at 25 000, but changing the absolute concentration of silk fibroin and Cu(II) in the solution (Fig. 5). As shown above, under this ratio, the $\log(A_0/A)$ value remained low indicating a high degree of complex formation between Cu(II) and silk fibroin macromolecules. Although the absolute amount of Cu(II) increased, the free Cu(II) evidently did not increase proportionally. Moreover, the slight decrease of $\log(A_0/A)$ value indicated more complex formation at higher concentrations. This could be understood as a shift in the equilibrium towards the product side when the reactant concentration increased.

3.4. Spinning mechanism and artificial spinning of silkworm silk

Some lepidopteran insects, including domesticated and wild silkworms, have evolved highly sophisticated protein polymers and spinning technology to produce very tough water resistant cocoons to protect their pupae [50]. The main selective pressure in this evolution has been that the pupal stage with its high energy content and lack of motility is highly vulnerable to predation, flooding and dehydration particularly during the long diapause in some species. Thus the remarkable properties of insect silks and the cocoons have provoked a great deal of interest in recent decades. This interest has been heightened by the recent finding that the silkworm silk can be produced with a comparable mechanical property to the exceptionally tough spider dragline silks simply by changing the spinning conditions of silkworm [11]. Though progress has been made in understanding the natural spinning technology, for instance the role of a liquid crystallinity [51,52] and a nucleation-dependent aggregation mechanism [53], our understanding of this highly complex process is far from complete. The evidence presented in this paper that cupric ions can form a complex with silk fibroin macromolecular chains inducing the conformation transition to β-sheet may have important implications for the spinning mechanism. Previous work [15,18] has suggested that other metallic ions including calcium and magnesium may also have important functions in the natural spinning process. However, it has been recently reported that the latter ions have no significant effect on the aggregation of regenerated silk fibroin [50]. This may be because the binding of these ions to native silk fibroin is conformation dependent while regenerated silk lacks these conformations as a result of its dissolution in powerful chaotropic solution. Our finding that cupric ions can induce the transition from random coil to β-sheet in regenerated silk fibroin as in other proteins, including PrP [39–46], has two important implications. First, it may provide a model system for studying the mechanism and kinetics of refolding with relevance to other proteins that undergo random coil to β-sheet, including the disease-causing prions. Finally this study reveals that trace quantities of a transition metallic ions may have more effect on silk fibroin than the alkali (Na^+, K^+) and alkaline earth metallic ions (Ca^{2+}, Mg^{2+}), with important implications for our understanding of the natural spinning process and artificial methods for spinning regenerated silk.

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