# Conformational Changes Induced in *Bombyx mori* Silk Fibroin by Cyclodextrin Inclusion Complexation

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ABSTRACT: We have previously demonstrated that the formation of and coalescence from polymer−cyclodextrin (CD) inclusion compounds (ICs) represents a very useful approach to modify the chain conformations and improve the crystallinity of various bulk polymers. The present work deals, for the first time, with the formation of a $\gamma$-CD IC with a natural protein as guest, i.e., silk fibroin from *Bombyx mori* silkworm. Formation of the crystalline inclusion compound was verified by wide-angle X-ray diffraction, solid-state NMR, and infrared spectroscopy to have the host $\gamma$-CD molecules arranged in a channel structure, with the isolated silk chains included, at least in *large* part, in their internal cavities. Removing the $\gamma$-CD host lattice by washing with hot water produced a white coalesced silk sample that was collected and characterized. Unlike the original or precipitated silk fibroin, the coalesced sample shows most of its protein residues in a $\beta$-sheet conformation with an elevated degree of crystallinity.

## Introduction

The textiles industry is in need of new materials with enhanced properties to replace or increase the usefulness of existing materials. One of the most valuable natural textile fibers is the silk protein from the *Bombyx mori* (*B. mori*) silkworm due to its exceptional mechanical properties. Also, because of its good mechanical properties and biocompatibility, *B. mori* silk fibroin (SF) has found increasing applications in biomedical materials.[1,2] However, to be more widely utilized, silk from the *B. mori* silkworm has to be regenerated and processed into appropriate forms.

Solid-state SF from *B. mori* is found in two different crystalline modifications, silk I and silk II, as well as the random coil form, depending on the conditions of sample preparation.[3] On the basis of X-ray diffraction studies, the conformation of silk II has been established as the antiparallel $\beta$-sheet form.[4] It appears that the less stable silk I is not as well-defined as silk II.[5,6] Silk II can easily be identified from the solid-state $^{13}$C NMR chemical shifts observed for glycine (Gly), serine (Ser), and alanine (Ala) residues, whereas the silk I form shows chemical shifts that are associated with a loose helical[7] or repeated $\beta$-turn[8,9] structure. A clearer distinction between these two forms can be made by FTIR spectroscopy or X-ray diffraction.

There have been several experimental attempts to modify or change SF from one crystalline form to another. For example, treatment of amorphous random coil SF films with organic solvents was investigated to determine whether the silk chains could be converted into silk I or silk II.[10] Similar conformational transitions were observed by immersion of SF films in water at different temperatures.[11] Also, silk films freshly cast from different solvents were found to be in an amorphous state and then subsequently transformed to $\beta$-sheet crystalline silk II by heating, solvent-induced crystallization, exposure to ultraviolet (UV) radiation, and prolonged storage.[12]

This work represents the first attempt to modify or change SF conformations and improve overall properties via formation of and coalescence from its inclusion compound (IC) with $\gamma$-cyclodextrin ($\gamma$-CD) host molecules. As we have demonstrated with many synthetic polymers, CD host molecules may accommodate, extend, and isolate single polymer chains in their *narrow* internal channels through formation of polymer−CD ICs.[13−20] Through full coverage of polymer chains with CD molecules, the long guest polymer molecules are forced to adopt highly straightened and extended conformations, which are at least partially retained after removal of CD molecules (coalescence) with a solvent good only for the host CD molecules. Here we describe, for the first time, the successful formation of a silk fibroin-$\gamma$-CD IC and the preliminary characterization of the silk sample coalesced from it.

## Experimental Section

Grade 5A raw silk was degummed and washed according to ref 21. Degummed SF was solubilized in a $Ca(NO_3)_2{\cdot}4H_2O$−2MeOH (Aldrich) solution at around 65 °C with vigorous stirring. A 10% w/v fibroin salt solution was dialyzed against deionized water, using a regenerated cellulose membrane (molecular weight cutoff of 6000−8000 Da), to reduce the salt concentration in the fibroin solution. The aqueous dialyzed solution of SF was transformed into a powder by freeze-drying.

Silk fibroin−$\gamma$-CD IC was obtained by precipitation of the two components in a common solution. 15 g of $\gamma$-CD powder (Cargill, Inc.) was first dissolved in 20 mL of a $Ca(NO_3)_2{\cdot}4H_2O$−2MeOH solution at 60° C using an ultrasonication bath. 1 g of degummed SF was then added to the clear warm $\gamma$-CD salt solution, while stirring vigorously for another 3 h at 60 °C. The resulting viscous and clear solution was allowed to cool to room temperature and slowly stirred for another 24 h before precipitating in methanol. The $\gamma$-CD/SF solution was added dropwise to highly stirred methanol (200 mL) at room temperature. The white precipitate was collected by vacuum filtration and then vacuum-dried overnight at 30 °C.

Control samples of $\gamma$-CD and SF were produced by precipitation of their individual salt solutions into methanol following all the above conditions. Other attempts to obtain a SF−$\gamma$-

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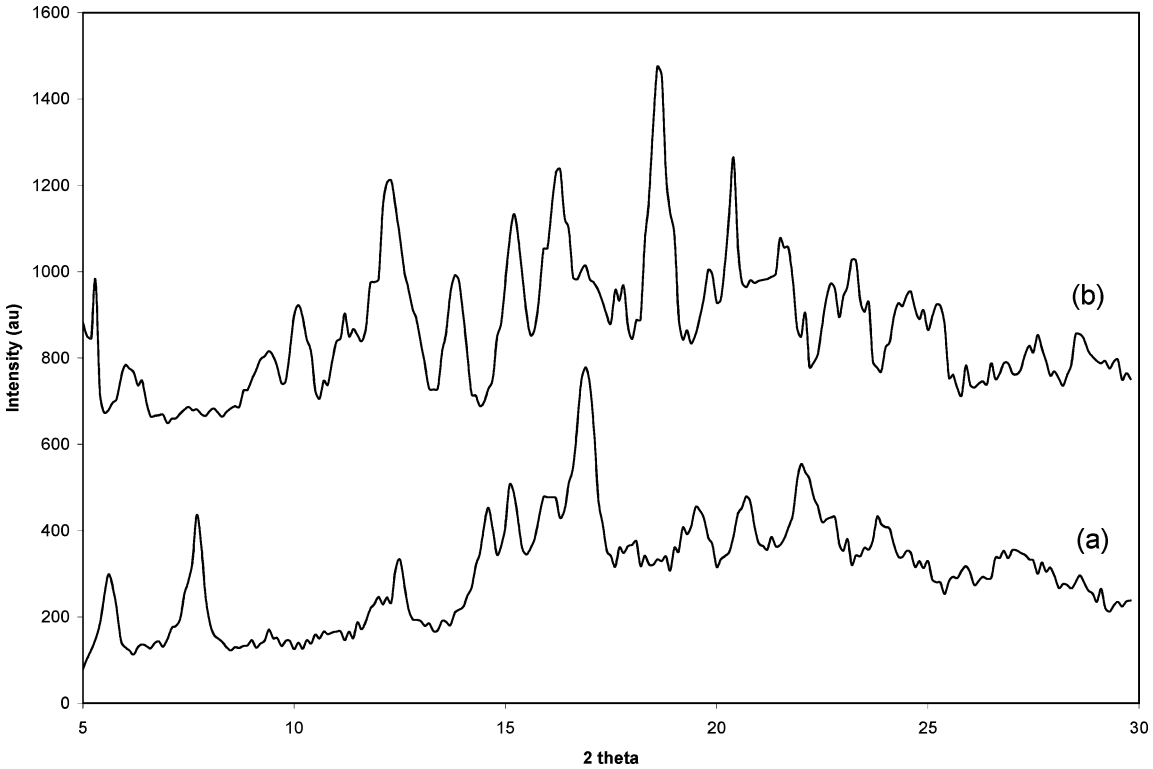


**Figure 1.** Wide-angle X-ray diffractograms of SF−$\gamma$-CD IC (a) and as-received $\gamma$-CD·7$H_2O$ (b).

CD IC, using DMSO/formic acid, DMSO/water, formic acid/formic acid, and DMSO/$Ca(NO_3)_2$·4$H_2O$−2MeOH as solvent systems for $\gamma$-CD/silk fibroin, failed. The freeze-dried and precipitated silks and SF−$\gamma$-CD IC were all powders and were observed directly by WAXD and solid-state CP/MAS $^{13}$C NMR. Native silk fiber and as-received $\gamma$-CD powder were used as control samples.

**Characterization**. *Wide-Angle X-ray Diffraction (WAXD).* Wide-angle X-ray diffraction (WAXD) measurements were performed with a Siemens type-F X-ray diffractometer equipped with a Ni-filtered Cu Kα radiation source ($\lambda$ = 1.54 Å). The diffraction intensities were measured every 0.1° from $2\theta$ = 5 to 30° at a rate of $2\theta$ = 3°/min. The supplied voltage and current were 30 kV and 20 mA, respectively.

*Solid-State $^{13}$C NMR Spectroscopy.* High-resolution solid-state $^{13}$C NMR experiments were carried out at 50.1 MHz on a Chemagnetics CMX200 spectrometer using cross-polarization and magic angle spinning (CP/MAS) with high-power proton dipolar decoupling (DD) of ∼47 kHz applied during acquisition. The spinning speed ranged from 4 to 4.2 kHz. $^{13}$C chemical shifts were referenced relative to TMS. Spectra were obtained with 1000 transients, 1.0 ms contact time, and 3.0 s pulse, or recycle, delay. The spectral width was 15 kHz in 2K data points, which were zero-filled to 8K before Fourier transformation.

*Fourier Transform Infrared Spectroscopy (FT-IR).* A Nicolet 510P FT-IR spectrometer was utilized to obtain the infrared spectra of samples mixed with potassium bromide (KBr) and pressed into pellets. The spectra were taken over a range of 4000−400 cm$^{-1}$ with a resolution of 2 cm$^{-1}$ using 64 scans.

## Results and Discussion

**Characterization of SF−$\gamma$-CD IC.** Several analytical tools, WAXD, NMR, and FTIR, were employed for the characterization of SF−$\gamma$-CD IC. For example, wide-angle X-ray diffractograms of the SF−$\gamma$-CD IC powder, as shown in Figure 1, indicate a channel structure that is completely different from that of the as-received $\gamma$-CD·7$H_2O$ in the cage structure. It is expected that once the host $\gamma$-CD molecules are threaded one by one onto the SF chains, the final IC structure can only be a channel structure with a columnar arrangement of host $\gamma$-CD s. As we and others have shown,[20,22,23] the intense diffraction peak at $2\theta \sim 7.5°$ is indicative of the columnar structure. In this $\gamma$-CD columnar structure, SF chains are forced to adopt a highly extended conformation, as they are included in the narrow $\gamma$-CD channels, and are segregated from neighboring protein chains by the walls of the $\gamma$-CD stacks. There is no evidence of any totally unincluded SF that might crystallize and show a peak at around $2\theta$ = 20°.

CP/MAS $^{13}$C NMR spectra were recorded for SF, $\gamma$-CD, and SF−$\gamma$-CD IC and are presented in Figure 2. The reduced splitting for the $\gamma$-CD carbon resonances in the IC crystals suggests a more mobile and conformationally disordered environment, thereby confirming the formation of the IC channel structure with the silk chains included. The three carbon resonance peaks corresponding to SF protein in the IC spectrum at 173, 50, and 44 ppm represent strong evidence of the presence of guest protein silk chains in the host $\gamma$-CD channels. Moreover, the weak intensity of these peaks correlates well with the small stoichiometric amount of SF that is expected to be included in the high molecular weight host $\gamma$-CD molecules. The presence of both host and guest molecules in the IC sample was also evidenced by FTIR spectroscopy (not shown here).

**Coalescence of the SF Chains from Their $\gamma$-CD IC Crystals.** All the above results strongly indicate that, if not all, then at least substantial portions of the SF protein chains are included and covered by the host $\gamma$-CD molecules. Coalescence of the SF was achieved by suspending the IC crystals in a large amount of warm water, which is a good solvent for the host CD molecules but not for the guest silk protein chains. The white, coalesced SF sample was collected by vacuum filtration and then vacuum-dried at 40 °C for 1 week. The yield

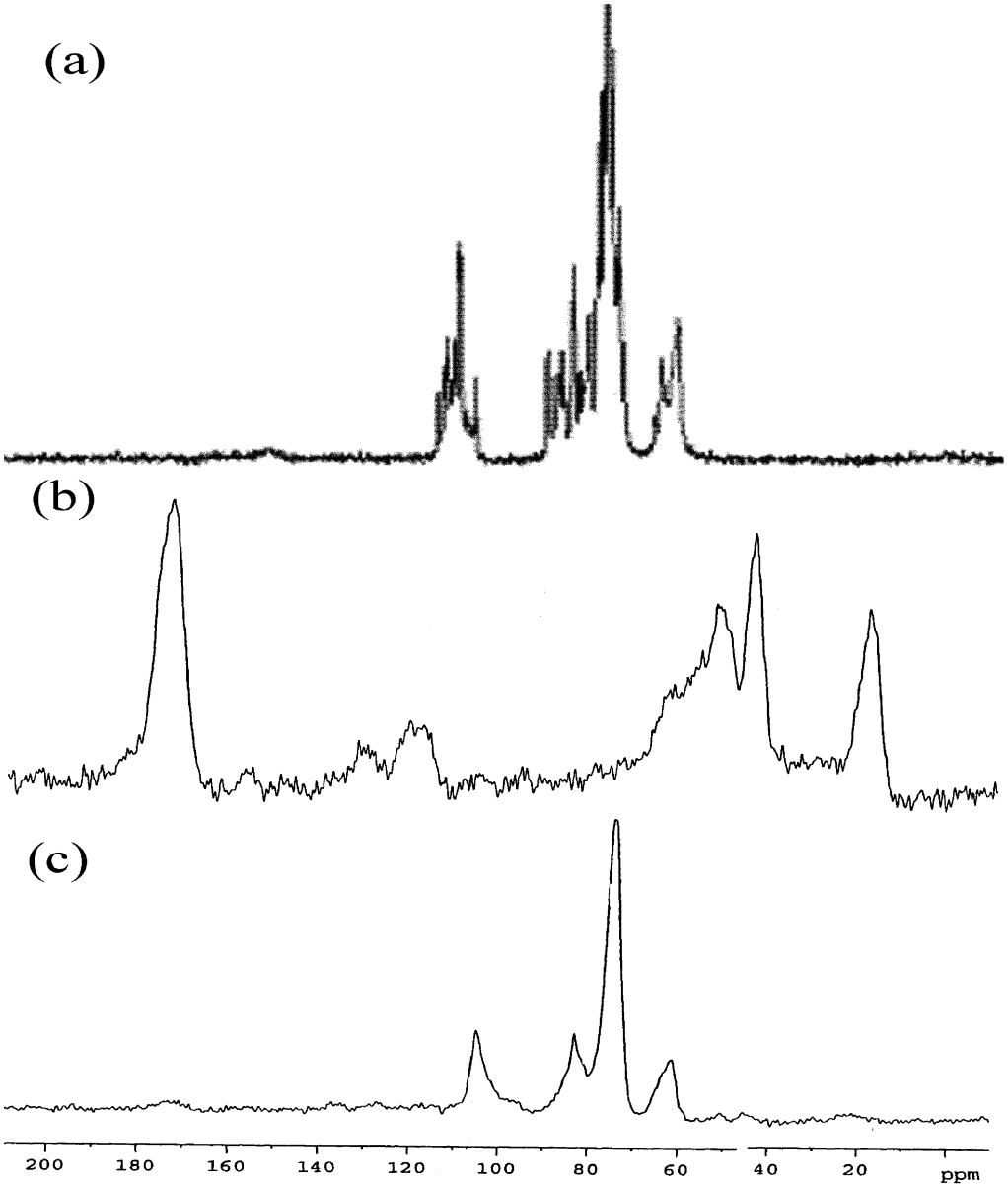


**Figure 2.** Solid-state $^{13}C$ NMR spectra of $\gamma$-CD (a), SF (b), and SF−$\gamma$-CD IC (c).

of coalesced SF was not inconsistent with the stiochiometry expected for SF−$\gamma$-CD IC. (Three amino acid units/$\gamma$-CD, ∼80 g/mol of amino acid, and mol wt of $\gamma$-CD = 1297 g/mol, so 3 × 80/1297 = 0.18 g SF:1.0 g $\gamma$-CD or ∼15 wt % SF is the expected stiochiometry for SF−$\gamma$-CD IC.)

*FTIR Spectroscopy*. Figure 3 presents a comparison of FTIR spectra recorded for the degummed, freeze-dried, and coalesced SF samples. As mentioned before, FTIR spectroscopy is very sensitive to the different crystalline modifications of silk. Each SF crystalline form shows a specific absorption band in four distinct vibrational regions associated with the amide groups in proteins. Amide I, II, and III bands are attributed to C=O stretching, N−H deformation, and O−C−N bending, respectively, whereas crystallinity is responsible for the amide V band, according to Bhatt and Ahirrao.[12] Two distinct bands have been associated with the amide I vibrations appearing at 1660 and 1630 $cm^{-1}$ as contributions from random coil and $\beta$-sheet conformations, respectively. FTIR analysis of this region clearly shows two peaks at 1704 and 1664 $cm^{-1}$, and a shoulder at 1641 $cm^{-1}$ for the degummed SF, an unique peak at 1650 $cm^{-1}$ for the freeze-dried SF, and one strong band at 1629 $cm^{-1}$, with a slight shoulder at 1704 $cm^{-1}$, for the coalesced SF. Therefore, the amide I band is assigned primarily to the random coil conformation for the degummed and freeze-dried SFs. The band position of coalesced SF in this region suggests a $\beta$-sheet conformation because C=O groups in $\beta$-sheets are involved in more H-bonds compared with randomly coiling conformations, shifting the corresponding FTIR band (amide I) to a lower frequency.

A similar observation can be made in the amide II region, where the peak at 1543 $cm^{-1}$ due to the random coil conformation is shifted to 1515 $cm^{-1}$ for the $\beta$-sheet conformation of SF. Both freeze-dried and degummed SF show one band at around 1540 $cm^{-1}$, confirming the existence of random coil conformation for these samples. Moreover, the degummed SF contains in this region another band at 1515 $cm^{-1}$, indicating the coexistence of some $\beta$-sheet conformation. By stretching and nanostructuring the original SF via $\gamma$-CD IC formation and coalescence, the random coil conformation is apparently

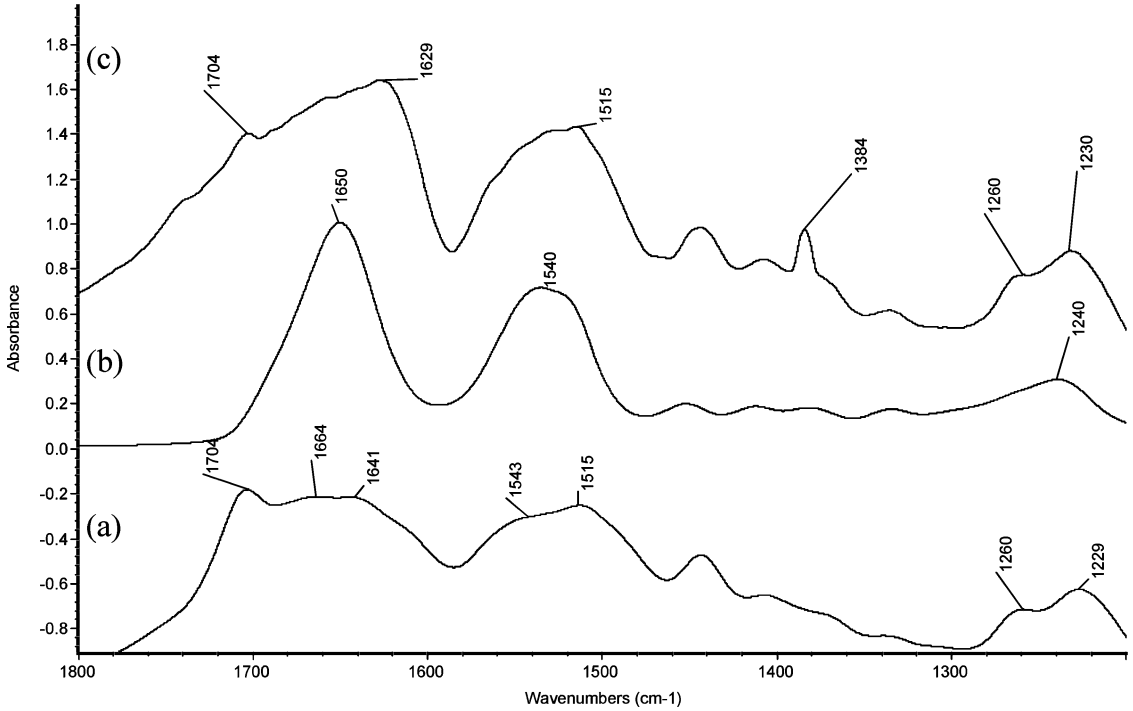


**Figure 3.** FTIR spectra recorded for degummed (a), freeze-dried (b), and coalesced SF (c) samples in the conformationally sensitive region between 1200 and 1800 $cm^{-1}$.

suppressed for the coalesced SF, a fact supported by the existence of a unique, but broad, band at 1515 $cm^{-1}$ for the $\beta$-sheet conformation.

The amide III band corresponding to the bending vibration of the O−C−N bond occurs in the range 1230−1260 $cm^{-1}$. The degummed and coalesced SF samples show two peaks in this region. The band at 1231 $cm^{-1}$ is due to the amorphous regions in the silk, whereas the band at 1260 $cm^{-1}$ is attributed to the crystalline phase (silk II or $\beta$-sheet) in these samples. The percentage of crystallinity in these two samples was calculated according to the Bhat and Ahirrao method[12] by using the intensity ratio of the bands at 1260 and 1230 $cm^{-1}$. The crystallinity index was calculated to be 0.65 for degummed SF versus 0.66 for coalesced SF. A possible reason for these similar crystallinities could be that the SF chains were not fully covered with $\gamma$-CD molecules during the inclusion process, and consequently, they were not entirely forced to change their conformations. In this regard, we should mention our results obtained for nylon-6 that was processed through the formation of and coalescence from its α-CD IC crystals.[17] In this case, full inclusion complexation was an effective approach to manipulate the nylon-6 crystalline structure from its as-received $\gamma$-form to its coalesced α-form, with a much higher level of crystallinity (80% increase) and substantially improved orientation of the extended nylon-6 chains in the coalesced sample.

The amide V band at 700 $cm^{-1}$ is attributable to crystalline conformations, as reported by Magoshi et al.,[11] while the presence of random coil conformations in this region is indicated by a band at 650 $cm^{-1}$. Degummed SF shows the coexistence of both types of conformations, while the coalesced sample unfortunately exhibits poor resolution in this IR region.

Another interesting peak is the symmetrical deformation band of Ala methyl groups, which occurs at 1385 $cm^{-1}$ and is suggested to be sensitive to both orientation and crystallization.[21] As expected, a weak band at 1384 $cm^{-1}$ is clearly observed for the coalesced SF as a consequence of its solidification from the narrow $\gamma$-CD channels in the silk−$\gamma$-CD IC.

*Wide-Angle X-ray Diffraction.* Figure 4 shows a comparison of the X-ray diffractograms recorded for the degummed, precipitated, and coalesced SFs. The original degummed silk sample shows a strong peak at $\sim 2\theta = 21°$, with a very weak shoulder around 25°, corresponding to the $\beta$-sheet crystalline form[21] and silk I, respectively. A similar X-ray pattern is observed for the coalesced and precipitated SFs, the latter (not shown) only after soaking in water for 15 h at 40 °C. The initial precipitated SF sample shown in Figure 4b was obtained from a solution of degummed SF in $Ca(NO_3)_2 \cdot 4H_2O$−2MeOH solvent by precipitation with methanol. This control SF sample was found to be almost completely amorphous. According to the literature,[11,12] the crystallinity and random coil conformation in SF can be changed by treatment with water and other solvents. To compare degrees of crystallinity, the precipitated SF was suspended in warm water (40 °C) for the same amount of time (15 h) as the coalesced SF that was recovered from its $\gamma$-CD IC by washing with warm water. Total crystallinities were estimated by taking the ratio of integrated peak intensities (areas) after and before subtraction of baseline intensities. The crystallinity of the initial precipitated SF was 14% and was enhanced to 39.2% after soaking in water. This value is still much lower than the crystallinity of coalesced SF, which is ∼60% when determined by the X-ray diffraction method.

This represents additional strong evidence confirming that CD IC processing offers an effective route for enhancing polymer crystallinity as well as for the manipulation of crystalline polymorphs.[24] The difference between crystallinity values estimated by the two analytical techniques (FTIR and X-ray) likely resides in the fact that IR is sensitive to short-range order,

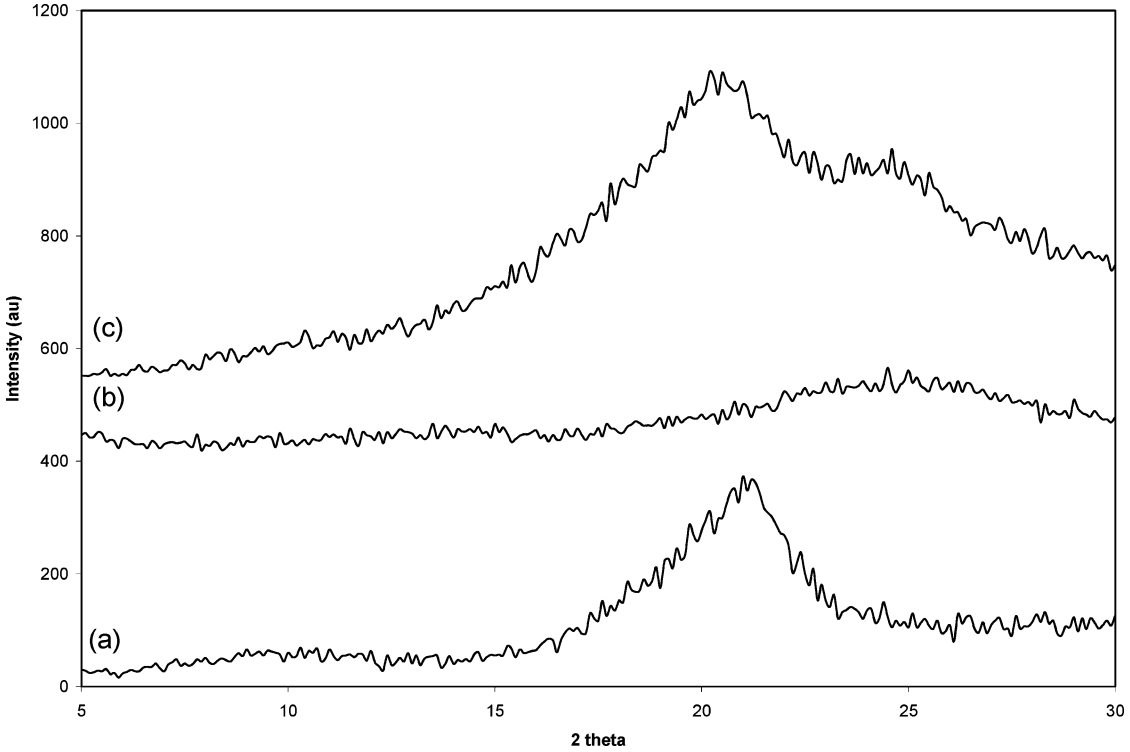


**Figure 4.** X-ray patterns of degummed (a), precipitated (before soaking) (b), and coalesced SF (c).

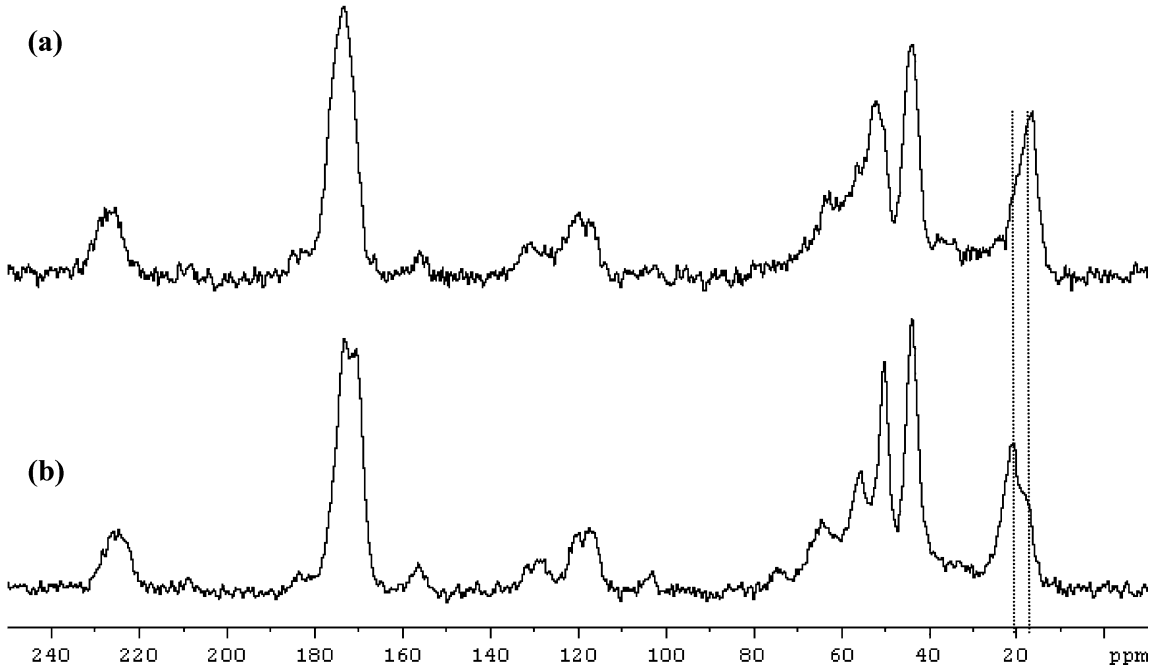


**Figure 5.** CP/MAS $^{13}C$ NMR spectra of precipitated (a) and coalesced SF (b). The two dotted lines drawn in the Ala $C_\beta$ portion of the spectrum at 20.0 and 16.4 ppm correspond[5] to silk II ($\beta$-sheet) and silk I (distorted $\beta$-turn) or random coil conformations in SF, respectively.

while X-ray diffractometry is sensitive to long-range order.[25]

*Solid-State $^{13}C$ NMR Spectroscopy.* To confirm the conformational differences between SF samples, CP/MAS $^{13}C$ NMR spectroscopy was performed, and the spectra of precipitated and coalesced SF are shown in Figure 5. The $^{13}C$ chemical shifts of SF indicated there for silk I or random coil are well displaced from those of silk II ($\beta$-sheet conformation), especially for the Ala methyl group (Ala $C_\beta$), and they can therefore be used for diagnostic purposes.[5,26,27] On the other hand, silk I and random-coil forms cannot easily be distinguished by $^{13}C$ NMR chemical shifts, but only by the extent of line broadening in the $^{13}C$ NMR peaks.[28–30] The main advantage of solid-state $^{13}C$ NMR spectroscopy results from its ability to observe the polymorphic structures of SFs without disruption of their ordered conformations by dissolution in solvents. A detailed literature search indicates accurate assignments for all peaks of the Gly, Ala, Ser, and Gln residues in SF, in both the $\beta$-sheet and silk I conformations.[5–7,28,29] On the basis of this collected data, we made the assignments for the Ala,

**Table 1. $^{13}C$ Chemical Shifts (ppm vs TMS) Observed in Solid Silk Fibroin Samples**

| | silk II ($\beta$-sheet)[5] | silk I[5] | precipitated SF | coalesced SF |
|---|---|---|---|---|
| Ala $C_\beta$ | 20.0 | 16.4 | 16.4 | 20.6, 17.3 |
| Ala $C_\alpha$ | 48.9 | 50.5 | 52 | 50 |
| Ala C=O | 172.5 | 174.1 | 173.2 | 173.2 |
| Gly $C_\alpha$ | 43.5 | 43.3 | 43.9 | 43.8 |
| Gly C=O | 169.8 | 172.7 | 173.2 | 170.5 |
| Ser $C_\alpha$ | 53.1 | 56.8 | 56 | 55.5 |
| Ser $C_\beta$ | 64.1 | 59.8 | 62.8 | 64.5 |

Gly, and Ser $^{13}C$ peaks observed in the NMR spectra of precipitated and coalesced SF and summarize them in Table 1. The carbonyl and α-carbons of Ala residues (Ala $C_\alpha$) in the silk I conformation are shifted downfield relative to those in a $\beta$-sheet, whereas the $\beta$-carbons (Ala $C_\beta$) are shifted upfield. $^{13}C$ peaks of the Gly $C_\alpha$ and Gly C=O are shifted upfield and downfield, respectively, for the silk I conformation, as compared to the $\beta$-sheet conformation. The $^{13}C$ NMR spectrum of the precipitated SF indicates just one peak in the C=O region at 173.2 ppm, which may correspond to both Ala and Gly residues. The peak positions of the Ala $C_\alpha$ at 52 ppm and Ala $C_\beta$ peak at 16.4 ppm are closer to the corresponding silk *I* values and demonstrate without any doubt that the Ala residues in the precipitated SF sample are present in a silk I (distorted $\beta$-turn) or random coil conformation.[28−30] Judging from the X-ray powder pattern (Figure 4b), the relatively broad Ala $C_\beta$ peak in the $^{13}C$ CP/MAS NMR spectrum (Figure 5a), and the $^{13}C$ chemical shift data in Table 1, our precipitated SF sample appears to adopt maily random coil conformations rather than the silk I conformation(s). Moreover, there is no evidence that any residue of the precipitated SF is present in the $\beta$-sheet conformation.

Judging from the chemical shifts of the coalesced SF peaks, some modifications in the conformations for certain residues may be suggested. An upfield shift of the $C_\alpha$ peak to 50 ppm and downfield shift of the $C_\beta$ peak to 20.6 ppm, relative to the random coiling/silk I precipitated SF, clearly indicate a $\beta$-sheet conformation for the Ala residues in the coalesced SF sample. However, the weak shoulder at 17.3 ppm, possibly a contribution of the Ala $C_\beta$ carbons in the silk I conformation, suggests the absence of a complete silk I to silk II conformational transformation for these residues. Asakura et al.[28] showed that by increasing the stretch ratio of *B. mori* silk fibroin fibers the relative intensity of the peak at around 19.9 ppm increases, and that at around 16.5 ppm simultaneously decreases. Comparing the relative intensities of Ala $C_\beta$ peaks in the coalesced and precipitated SF samples, we may conclude that the $\gamma$-CD IC formation and coalescence processes helped to extend and therefore to change the conformation of SF from silk I to predominantly silk II.

At first glance, the possibility that $\gamma$-CDs (9−10 Å channel diameter) have completely threaded over SF protein chains to form the SF−$\gamma$-CD IC seems unlikely due to the presence of bulky side chains on some of the amino acids (Pro, His, Trp) found in the SF protein, including some at both ends of the SF heavy chain.[31] However, there is precedence for such an observation in the study of the complexation of propylene oxide-*b*-ethylene oxide-*b*-propylene oxide (PPO−PEO−PPO) block copolymer with α-CD.[32] As pointed out in this study, PPO and PEO homopolymers are known to form CD ICs only with $\beta$- and α-CDs, respectively. Nevertheless, it was observed there that α-CDs are able to thread over the bulkier PPO end blocks and complex with the central PEO block. In an analgous manner, $\gamma$-CDs are apparently able to thread over the bulky amino acid residues in the SF protein chains and form a SF−$\gamma$-CD IC.

Also, Asakura demonstrated the presence of two kinds of structures in silk II: structure A (all methyl groups of Ala residues point toward the same direction in adjacent $\beta$-sheets) assigned to the 22.2 ppm peak and structure B (Ala methyl groups alternately point in opposite directions in the adjacent $\beta$-sheets) assigned to the 19.9 ppm peak.[28] It is noted that the peak at 20.6 ppm is very broad in Figure 5b, which also suggests the appearance of heterogeneous silk form II structures in our coalesced SF.

The right-hand shoulder of the split resonance at 170.5 ppm corresponds to the C=O peak of Gly residues in coalesced SF, which are found to be in the $\beta$-sheet conformation. Moreover, the chemical shifts observed for the Ser residues of the coalesced SF sample at 55.5 and 64.5 ppm are shifted upfield and downfield, respectively, in comparison with the corresponding peaks of the precipitated SF, and are much closer to the values for the $\beta$-sheet conformation.

It is also noteworthy that the $^{13}C$ NMR signals of Gly, Ala, and Ser residues are well resolved and narrower in coalesced SF compared with those of precipitated SF. This might be a consequence of a smaller content of random coil conformation in the coalesced SF, which tends to broaden the NMR signals[6] and also suggests a better crystalline packing in the coalesced SF. Thus, in summary, most of the coalesced SF chains are in $\beta$-sheet conformation, with a few residues remaining in the silk I form, most probably because of an incomplete coverage of the original SF chains with the host $\gamma$-CD molecules.

## Conclusions

We report here, for the first time, formation of a SF−$\gamma$-CD IC followed by the coalescence of included and conformationally restricted silk chains. All employed analytical analyses confirmed the successful formation of a crystalline channel arrangement of host $\gamma$-CDs with SF chains included in an extended conformation in their columnar cavities. Coalescence of the nanostructured SF chains, by washing the CD molecules away with warm water, leads to suppression of the silk I and/or randomly coiling entangled conformations in the resulting solid SF sample, which now contains a much higher quantity of $\beta$-sheet conformation. FTIR, WAXD, and solid-state $^{13}C$ NMR analyses indicate a higher degree of crystallinity and more orientated chains for the coalesced SF in comparison with the initial degummed or precipitated SF samples, and these modifications may have a substantial impact on the overall physical properties of $\gamma$-CD-processed silk. As a consequence, $\gamma$-CD IC formation/coalescence processing has proved to be an effective method of manipulating both the polymorphic structures and the final level of crystallinity in the SF protein.

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