Distinctive Influence of Two Hexafluoro Solvents on the Structural Stabilization of *Bombyx mori* Silk Fibroin Protein and Its Derived Peptides: ^{13}C NMR and CD Studies

Sung-Won Ha,[†] Tetsuo Asakura,*[,†] and Raghuvansh Kishore[‡]

Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan, and Institute of Microbial Technology, Sector 39-A, Chandigarh - 160 036, India

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Employing high-resolution ^{13}C solution NMR and circular dichroism (CD) spectroscopic techniques, the distinctive influence of two intimately related hexafluoro solvents, 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and hexafluoroacetone trihydrate (HFA), on the structural characteristics of *Bombyx mori* (*B. mori*) silk fibroin, the chymotrypsin precipitate (C_p) fraction, and two synthetic peptides, $(AGSGAG)_5$ and $(AG)_{15}$, is described. The observed ^{13}C solution NMR and CD spectra of these polypeptides in HFIP and HFA revealed a distinctive influence on their conformational characteristics. The ^{13}C NMR spectra, as analyzed from the unique chemical shifts of C_α and C_β resonances of constituent residues revealed that fibroin largely assumes helical conformation(s) in both solvents. However, the peak shifts were greater for the samples in HFIP, indicating that the types of helical structure(s) may be different from the one populated in HFA. Similar structural tendencies of these polypeptides were reflected in CD spectra. The observed CD patterns, i.e., a strong positive band at ∼190 nm and negative bands at ∼206 and 222 nm, have been attributed to the preponderance of helical structures. Of the two prevalent helical structures, α-helix and 3_{10}-helix, the evidence emerged for the fibroin protein in favor of 3_{10}-helical structure stabilization in HFIP and its significant disruption in HFA, as deduced from the characteristic $R1$ ($=[\theta]_{190}/[\theta]_{202}$) and $R2$ ($=[\theta]_{222}/[\theta]_{206}$) ratios, determined from the CD data. Conversely, the native polypeptides and synthetic peptide fragments derived from highly crystalline regions of the silk fibroin protein sustained predominantly an unordered structure in HFA solvent.

Introduction

The well-known fibrous protein produced by the domestic silkworm *B. mori*, the silk fibroin, has a number of outstanding properties not only for textile applications (e.g., luster, comfort, high strength, elasticity, dyeability, etc.) but also for design and development of various biotechnological and biomedical devices.[1−4] Extensive exploitation of *B. mori* fibrous protein essentially originates from its unusual amino acid composition, which is translated into an extraordinary primary structure and hierarchical structural organization.[5−7] The superior nature of the silk fibroin makes it an excellent natural fiber, which presumably originates from the precise self-organization of the unique repetitive sequences.[5−7]

Previously, Trabbic and Yager[8] and Asakura and co-workers[9] independently attempted an appraisal of the relative merits of two hexafluoro solvents, HFIP and HFA, respectively, for synthetically spun fibers from *B. mori* silk fibroin. The reported characteristic variations in the physicochemical properties of the regenerated silk fibers prepared from the two fluorinated solvents probably originate from their indistinct primitive molecular structures in solution. To understand and validate the conjecture that distinct primitive molecular conformations of the silk fibroin in hexafluoro solvents might remarkably influence the physical characteristics of regenerated silk fibers, we intend to analyze and compare the structural properties of the native silk fibroin and the derived peptides mimicking the highly crystalline regions of the protein.

Employing high-resolution ^{13}C solution NMR and CD techniques, in the present paper, we report the solution structures and the extent of folding−unfolding behavior of the fibroin molecule in pure HFIP and HFA solvents. Moreover, to establish the precise role played by the prevalent unique hexameric sequence motif (GAGAGS), the C_p fraction[10] (GAGAGSGAA-$[SGAGAG]_n$Y, where n ranges between 6 and 10) and the two synthetic model peptides, $(AGSGAG)_5$ mimicking the highly repetitive crystalline region of the silk fibroin and $(AG)_{15}$ giving silk I X-ray diffraction pattern after appropriate treatments, are also examined. Therefore, in view of excellent textile and diverse biotechnological applications of silk-based biomaterials, we are inclined by the results to point out that distinct variations in the preferred primitive secondary structures in hexafluro solvents possibly contribute to specific physical properties of synthetically spun *B. mori* silk fibers.

Materials and Methods

***B. mori* Silk Fibroin.** The silk fibroin used in this study was prepared according to the procedure employed previously.[11−13] Briefly, *B. mori* cocoons were cut in small pieces and degummed in a mixture of sodium carbonate (0.25% w/v) and Marseille soap (0.25% w/v) solution at 85 °C for 15 min. After washing and drying, degummed silk fiber was dissolved in 9 M aqueous LiBr solution and then dialyzed against deionized water for 3 days. Dialyzed aqueous fibroin solution was freeze-dried for further use.

***C_p* Fraction of *B. mori* Silk Fibroin.** The fraction of *B. mori* silk fibroin precipitated after chymotrypsin enzymatic cleavage was obtained

* To whom correspondence should be addressed. Tel & Fax: +81-42-383-7733. E-mail: asakura@cc.tuat.ac.jp.
[†] Tokyo University of Agriculture and Technology.
[‡] Institute of Microbial Technology.

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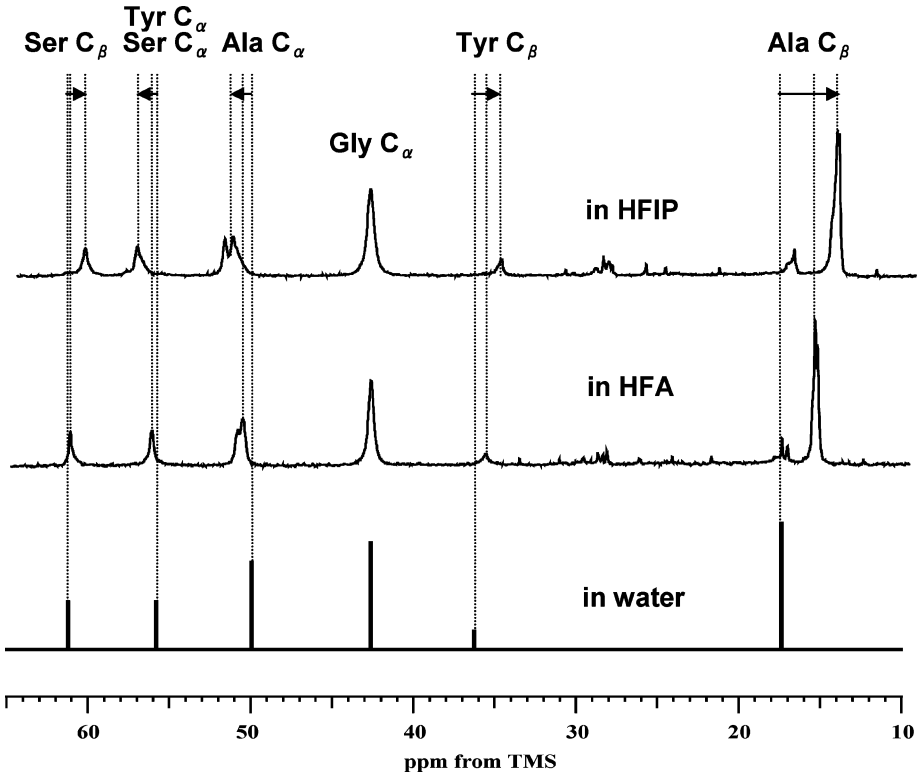

Figure 1. Expanded high-resolution ^{13}C solution NMR spectra (C_α and C_β regions) of *B. mori* silk fibroin dissolved in water, HFA, and HFIP. The stick spectrum of the fibroin dissolved in water was regenerated from our previous report.[15]

according to the method described earlier.[12,13] Briefly, the C_p fraction was prepared from chymotrypsin (40 mg, Seikagaku Kogyo Co., Japan), dissolved in a few milliliters of water, and added to an aqueous solution of about 4 g of regenerated silk fibroin buffered with $Na_2HPO_4 \cdot 12H_2O$ and $NaH_2PO_4 \cdot 2H_2O$ at pH 7.8. The solution was incubated at 37 °C for 24 h and then washed with 0.03 N HCl to inactivate the enzymatic reaction. The precipitate was separated by centrifugation and washed repeatedly with distilled water, ethyl alcohol, and diethyl ether, and then freeze-dried.

Peptide Synthesis. The chemical synthesis of two model peptides, $(AGSGAG)_5$ and $(AG)_{15}$, was achieved by employing standard solid-phase methodology, using Fmoc chemistry, on a fully automated Pioneer peptide synthesis system.[14] The peptide was assembled on Fmoc-Gly-PEG-PS resin (0.19 mmol/g), and the coupling of Fmoc amino acids was performed by HATU (*N*-[(dimethylamino)-1*H*-1,2,3-triazaolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide). After completing the synthesis, peptides were released from the resin by treating the peptidyl resin with a mixture of trifluoroacetic acid, phenol, triisopropylsilane, and water (88:5:2:5 in vol %) for 2 h at room temperature. The crude peptides were dissolved in 9 M aqueous LiBr solution and dialyzed against deionized water for the purification. After the dialysis, the precipitate collected by centrifugation was dried in a vacuum. Both peptides were recovered as a white powder by filtration and used directly for CD experiments.

^{13}C Solution NMR. The ^{13}C solution NMR experiments were performed at 25 °C with JEOL α-500 NMR spectrometer operating at 125 MHz for ^{13}C nucleus. The 45° pulse with an acquisition time of 1 s and a delay time of 2.5 s was used for the observation. The spectral width was 34 kHz with data points of 32 k. Up to 12 k scans were accumulated. ^{13}C chemical shifts were calibrated through an internal dioxane peak observed at 66.5 ppm relative to tetramethylsilane at 0.0 ppm. The sample concentration was 4% (w/w), and a small amount of D_2O (5%) was added to the sample solution to obtain a deuterium lock signal.

Circular Dichroism. CD spectra (185−260 nm) of all the samples were recorded on a JASCO J-805 spectropolarimeter, at an ambient temperature of 25 °C. A cylindrical quartz cell of 0.01 cm was used for spectral measurements. 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: $[\theta]_M \times 10^{-3}$ (deg·cm^2·dmol^{-1}), based on the molecular weight of the protein and peptide samples (*B. mori* fibroin ≈ 400 kDa, C_p fraction ≈ 3980 g/mol assuming $n = 8$, $(AGSGAG)_5 = 2018$ g/mol, and $(AG)_{15} = 1938$ g/mol). The two fluorinated solvents, HFIP and HFA, were purchased from Wako Pure Chemical Industries Ltd., Japan, and Sigma-Aldrich, U.S.A., respectively.

Results and Discussion

The ^{13}C solution NMR spectra of the *B. mori* silk fibroin molecule, dissolved in water, HFA, and HFIP, are shown in Figure 1. The stick spectrum of the fibroin dissolved in water was regenerated from our previous report.[15] From the analysis of the conformation-dependent ^{13}C chemical shift of C_α and C_β resonances in peptides and proteins, it is generally accepted that these values shift to lower and higher magnetic fields, respectively, as the conformation changes from β-sheet to random coil to helix.[16,17] For example, the typical chemical shifts of the conformation-sensitive Ala C_α and C_β resonances for solid silk fibroin samples, in helical- and β-sheet structures, are reported to be ∼52.0, 15.0 and 48.0, 20.0 ppm, respectively. Upon changing the solvent from aqueous to nonaqueous fluorinated medium, it is clear that the chemical shifts of C_α and C_β resonances of Ala, Ser, and Tyr residues are shifted to lower and higher magnetic fields, respectively, for *B. mori* silk fibroin dissolved in HFA and HFIP compared to those in water. Fibroin molecules dissolved in an aqueous system have been known to adopt either the random coil or silk I structure.[13,15] From the chemical shift values of C_α and C_β, the conformation of the fibroin molecule appears to be predominantly helical on changing the solvent to hexafluorinated systems, e.g., 50.8 and

Table 1. The CD Spectral Characteristics of *B. mori* Silk Fibroin, C_p Fraction, and Model Peptides, $(AGSGAG)_5$ and $(AG)_{15}$, in HFIP and HFA

solvents		molar ellipticity ($[\theta]_M$), *R*1 & *R*2	*B. mori* silk fibroin	C_p fraction[a]	$(AGSGAG)_5$	$(AG)_{15}$
HFIP		$\lambda_{max} \approx 190$ nm	70 255	314	83	−137
	$[\theta]_M$	$\lambda_{min} \approx 198-206$ nm	−47 033	−239	−147	−218
		$\lambda_{min} \approx 222$ nm	−22 843	−104	−62	−44
		$R1 = ([\theta]_{190}/[\theta]_{202})$	−1.49	−1.31	−0.56	0.63
		$R2 = ([\theta]_{222}/[\theta]_{206})$	0.49	0.44	0.42	0.20
HFA		$\lambda_{max} \approx 190$ nm	30 094			
	$[\theta]_M$	$\lambda_{min} \approx 198-206$ nm	−23 298	−86	−107	−154
		$\lambda_{min} \approx 222$ nm	−18 817	−34	−33	−39
		$R1 = ([\theta]_{190}/[\theta]_{202})$	−1.29			
		$R2 = ([\theta]_{222}/[\theta]_{206})$	0.81	0.40	0.31	0.25

[a] C_p fraction:[10] GAGAGSGAA[SGAGAG]$_n$Y, where *n* ranges between 6 and 10. *n* is assumed to be 8 for the calculation of molecular weight of the C_p fraction.

51.1 ppm for Ala C_α, 15.8 ppm for Ala C_β in HFA; 51.8 and 52.3 ppm for Ala C_α, 14.6 ppm for Ala C_β in HFIP. Moreover, from the fact that the peak shifts were greater for the sample in HFIP, it is obvious that the highly ordered helical contents increased more in HFIP than in HFA. Alternatively, there may be a possibility that the ordered and nonextended fibroin conformation is different in these two hexafouoro solvents. It is well-established that the Ala, Ser, and Tyr residues are the dominant residues that comprise the crystalline (GAGAGS) and semicrystalline (GAGAGY) regions of *B. mori* silk fiber. Therefore, from the NMR results, it can be speculated that the helical structures in the two fluorinated solvents are associated with both crystalline and semicrystalline regions, although their natures may be altered. The result leads us to infer that the effective interactions between the hydrophobic crystalline and semicrystalline regions in *B. mori* silk fibroin and the two fluorinated solvents might be altered. The other minor amino acids, such as charged, bulky, and hydrophilic moieties, occur in the irregular or nonrepeating sequences of the *B. mori* silk fibroin heavy chain;[5−7] the ^{13}C chemical shifts of C_β are shifted to higher fields in both HFIP and HFA as compared to those in aqueous environment. Even though the irregular or nonrepeating sequences take a helical conformation in both solvents, we presume similar interactions with the two hexafluoro solvent molecules. Collectively, the results indicate that the influence of HFA and HFIP on the preferred molecular structures of the fibroin molecule is unlikely to be dependent on the hydrophobicity and/or hydrophilicity of the residues alone. To provide further support and to substantiate the findings inferred by the NMR studies, chiroptical investigations were performed in HFA and HFIP and compared with the data described elsewhere in aqueous solution.

Figures 2 compares the far-UV CD spectra of *B. mori* silk fibroin, the C_p fraction, and two synthetic model peptides, $(AGSGAG)_5$ and $(AG)_{15}$, in HFIP and HFA. Table 1 summarizes the CD spectral characteristics of the CD curves shown in Figure 2. The observed CD spectrum of the silk fibroin molecule in HFIP (Figure 2a) is characterized by the appearance of a prominent double minimum at ∼206 and ∼222 nm and a strong positive band at ∼190 nm. The spectral pattern provides a strong indication favoring the existence of a right-handed helical conformation, albeit not readily identifiable to a classical α-helix.[18−22] On the other hand, the spectral features of the fibroin protein in HFA are remarkably altered. In this solvent, the fibroin exhibits the characteristic double minima at ∼222 and ∼205 nm of almost similar intensity and an intense positive band at ∼198 nm. From the spectral pattern, it seems apparent that even in HFA the fibroin molecule prefers a helical conformation, although different from the one perceived in HFIP. It is noteworthy that the first report in the literature highlighted the role of HFA hydrate as a "helix-breaking" solvent for polypeptides and proteins.[23] Interestingly, while characterizing a molten globule-like state of hen eggwhite lysozyme in 25% aqueous HFA hydrate by CD in conjunction with fluorescence and NMR techniques, Balaram and co-workers observed a second structural transition from the compact molten globule-like state to a noncompact open helical state at higher concentration of HFA (≥50%).[24]

To validate that the preferred regular secondary structures of *B. mori* silk fibroin in HFIP and HFA are primarily dictated by its highly repetitive sequence motifs, CD spectra of the C_p fraction, GAGAGSGAA[SGAGAG]$_n$Y (*n* may vary from 6 to 10),[10] and two synthetic model peptides represented by $(AGSGAG)_5$ and $(AG)_{15}$, were measured (see Figure 2b−d). Although the overall CD spectral patterns of the C_p fraction in HFIP are remarkably similar to the one observed for the silk fibroin, nevertheless a substantial reduction in the corresponding band intensities was observed. Perceptibly, the CD spectra of $(AGSGAG)_5$ and $(AG)_{15}$ in HFIP show distinct characteristics, i.e., while the $(AGSGAG)_5$ sequence appears to be predominantly helical, the lack of Ser residues in $(AG)_{15}$ strongly disrupts the helical structure. The results clearly suggest that the periodic presence of the short polar hydroxyl groups of the Ser residues may specifically interact with HFIP molecules, favoring induction and/or stabilization of helical structures. The relatively higher content of Ser residues in the C_p fraction, as compared to $(AGSGAG)_5$ sequence, apparently substantiates the conjecture. It is noteworthy that the CD spectral pattern of the C_p fraction in HFIP is very similar to that of fibroin protein. The interactions become greater as the fibroin protein contains more Ser residues. Therefore, the observed CD spectra of silk fibroin, C_p fraction, $(AGSGAG)_5$ and $(AG)_{15}$ in HFIP solution, lead us to suggest the existence of an additional mechanism for the helix formation, except for the "Teflon-coat" effect hypothesized by Balaram and co-workers.

For the samples in HFA, the intensity minima at ∼195 nm are shifting to higher wavelengths up to 205 nm, as the sample contains more hydrophilic amino acids, i.e., from $(AG)_{15}$ to *B. mori* silk fibroin, indicating that the random coil characteristic is decreasing. Nevertheless, the CD spectra of the C_p fraction, $(AGSGAG)_5$, and $(AG)_{15}$ in HFA clearly show the absence of defined regular molecular structure(s), presumably indicating the helix-destabilizing effects exerted by HFA molecules.

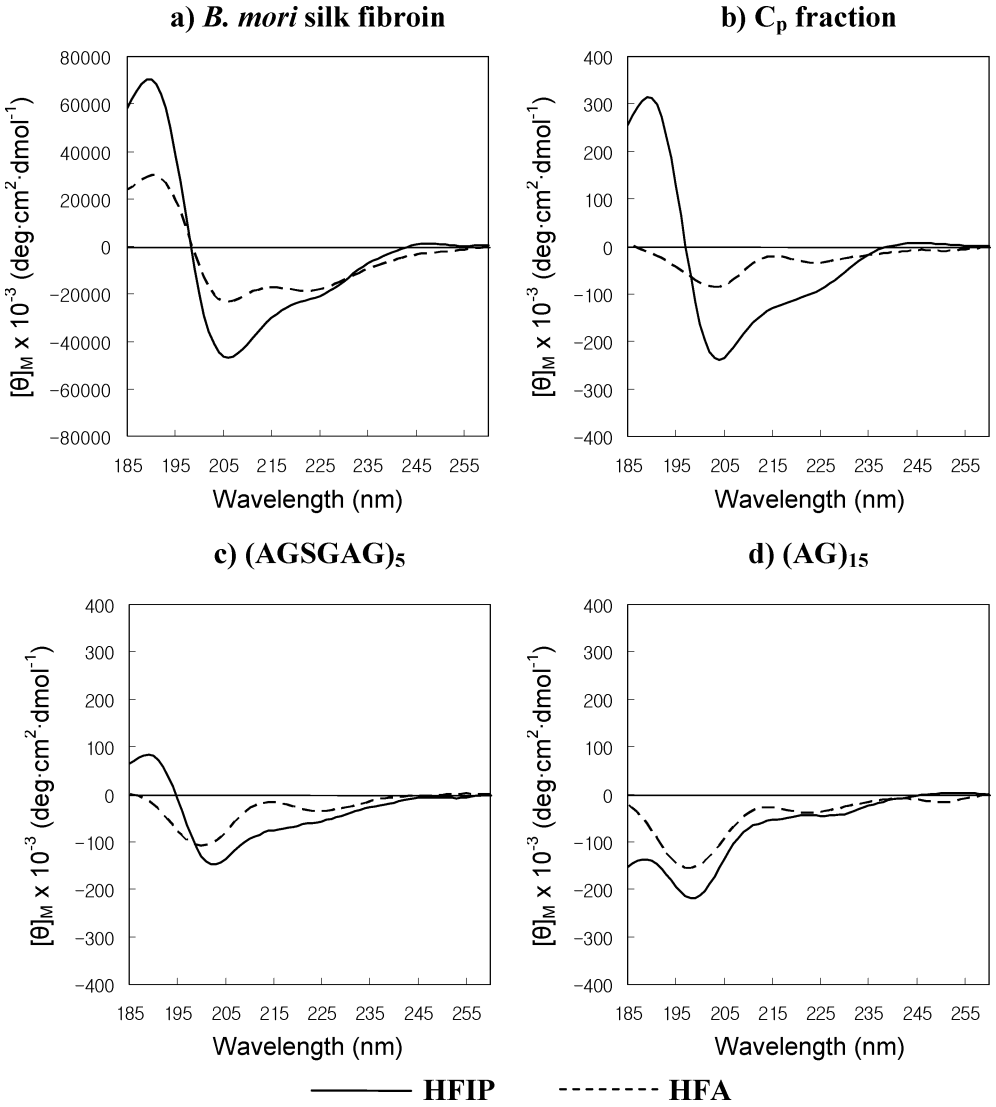

Figure 2. The observed CD spectra of *B. mori* silk fibroin (a); the C_p fraction (GAGAGSGAA[SGAGAG]$_n$Y *n* may range from 6 to 10) (b); $(AGSGAG)_5$ (c), and $(AG)_{15}$ (d) in HFIP (—) and HFA (- - -).

Previous investigations have described the characteristic of *R*1 (the ratio between the intensity of the maximum between 190 and 195 nm and the intensity of the minimum between 200 and 210 nm, i.e., $R1 = [\theta]_{190}/[\theta]_{202}$) and *R*2 (the ratio between the intensity of the minimum near 222 nm and the intensity of the minimum between 200 and 210 nm, i.e., $R2 = [\theta]_{222}/[\theta]_{208}$) values for distinguishing the nature of common helical structures in proteins and polypeptides, including fibrous proteins and the derived peptide sequences. The fibroin and C_p fraction in HFIP tend to assume a unique right-handed helical conformation, other than an α-helix. The previous reports demonstrated the potential utility of *R*1 and *R*2 parameters and received experimental verification for differentiating the two intimately related helical structures, right-handed α- and 3_{10}-helical structures, and are suggested to be in good agreement with theoretical calculations.[26−30] On the basis of X-ray diffraction, ^{1}H NMR, and IR spectroscopic analyses of terminally protected homo-octamers, *Z*-[L-(α-Me)Val]$_8$−O*t*Bu, Toniolo et al., strongly argued in support of CD data (*R*2 ratio being 0.4 in TFE), favoring the existence of the right-handed 3_{10}-helical structure in solution.[29] It is noteworthy that short synthetic peptides incorporating nonproteinogenic $C^{\alpha,\alpha}$-disubstituted glycines severely restrict the backbone conformations in the 3_{10}-helical regions of the Ramachndran map.[31,32] In this context, it must be highlighted that *B. mori* silk fibroin, established to be a *β*-sheet containing fibrous protein, has a rich content of achiral proteinogenic Gly residues (∼43%). However, it exhibits a strong inclination for the right-handed 3_{10}-helical structure only in HFIP. Further indication for the fact that silk fibroin is capable of adopting primarily an unusual helical conformation in HFIP was received from IR, UV, optical rotatory dispersion (ORD), and CD spectroscopic studies performed on three poly-tripeptides, poly(Ala-Ala-N^{ϵ}-carbobenzoxy-Lys), poly(Ala-Ala-Lys-hydrobromide), and poly(Ala-Ala-Gly), and poly(Ala).[33] Although Parrish and Blout reported surprisingly comparable CD spectral patterns for the three poly-tripeptides and poly(Ala) in HFIP, the possibility for the existence of the 3_{10}-helical structure was presumed to be implausible. Surprisingly, these authors pointed out the existence of a new form of helix, and the proposed backbone torsion angles ($\phi \approx -65°$ and $\psi \approx -40°$) lie somewhere between the two forms of α-helical structures: the α_I-helix (classical α-helix) and the α_{II}-helix (with no hydrogen bonds).[33] In addition, the proposed helical structure facilitated the formation of two hydrogen bonds with each intrahelix carbonyl group, i.e., a doubly hydrogen-bonded helical structure. It is worth mentioning that, like the high-molecular-weight poly-tripeptides and poly(Ala), the silk fibroin is also rich in Ala content (∼30%), besides Gly residues (∼43%), and

the preponderance of GA sequence motifs in the crystalline regions possibly generate unique hydrophobic stretches along the silk fibroin chain.

The observed CD patterns of the silk fibroin in pure hexafluoro solvents clearly preclude the existence of an extended β-sheet ($\phi \approx -140°$, $\psi \approx 135°$) and left-handed poly(L-Pro) type II (PPII) helical ($\phi \approx -80°$, $\psi \approx 150°$) structures, stabilized by interchain hydrogen-bonding interactions and, therefore, are not considered for discussion.[18−22,34−37] Considering the possibility of other well-defined helical structures, frequently observed in proteins and polypeptides, the CD spectral features of the fibroin and its derived peptides in HFIP may apparently be assigned to either of the two closely related helical structures, α-helix ($\phi \approx -60°$ and $\psi \approx -45°$) or 3_{10}-helix ($\phi \approx -60°$ and $\psi \approx -30°$), stabilized by intrahelical hydrogen bonds.[25−28,37] In view of theoretical as well as experimental aspects of CD spectral analyses, there exists the possibility of distinguishing the α- and 3_{10}-helical structures.[26−30,38] The *R*1 and *R*2 ratios could be the possible differentiating parameters for the two helical conformations. For the predominant 3_{10}-helical structure, the characteristic *R*1 and *R*2 values are expected to approach −1.0 and 0.5, respectively, whereas the corresponding values should approach −2.0 and 1.0 in the case of typical α-helical structure. Consistent with ^{13}C NMR and CD spectral data, the silk fibroin in aqueous solution is predominantly an unordered structure, i.e., the *R*2 ratio is expected to approach zero.[26−30,38] As is evident from Table 1, the determined *R*1 and *R*2 values for the silk fibroin in HFA are −1.3 and 0.8, respectively, indicative of largely right-handed α-helical type conformation, whereas those in HFIP approach −1.5 and 0.5, respectively, suggestive of a significant population of 3_{10}-helical structure. The discrepancy in *R*1 values is probably due to the dramatic increase in high tension (HT) in the wavelength between 195 and 185 nm as well as fibroin's conformational variability in the solution state. Overall, these data fail to substantiate the conclusion drawn by Trabbic and Yager: In pure HFIP, the silk fibroin is presumed to be predominantly α-helical (∼50% α-helix and 24% β-sheet).[8]

Analysis of ^{13}C solution NMR and CD spectral data of *B. mori* silk fibroin, dissolved in HFIP and HFA, reveal that two fluorinated solvents exert distinctive influence on the appearance of molecular structures. We propose that the preferred primitive conformation, characterized in the specific solvent, may influence the improved physical characteristics of the regenerated silk fibers, although the regenerated silk fibers obtained from the HFIP or HFA trihydrate solvent system under appropriate processing conditions captured the important features of the native fibers.[8,9] The molecular structure of the silk fibroin in HFIP is suggested to be predominantly α-helical (α-helix ≈ 50% and β-sheet ≈ 24%), which is largely at variance from our results. Spinning the solution to form an undrawn fiber remarkably changed the relative secondary structural contents.[8] On the other hand, high-resolution ^{13}C CP/MAS NMR spectral analysis of the artificially spun silk fibers obtained from HFA, after postspinning drawing and steam-annealing at higher temperature (≥100 °C), revealed that regenerated silk fiber captured the main features of the native fibers, i.e., it adopts the β-sheet structure. Even though other spinning and postspinning conditions are comparable, our current artificial spinning results (data not shown here) show that a much stronger fiber can be regenerated from HFIP dope than from HFA dope, whose tensile properties are comparable to those of native *B. mori* silk fiber. Speculatively, fibroin molecules with stretched and tightly wound 3_{10}-helical structure in HFIP may be better for the construction of improved crystalline orientation during the spinning and postspinning processes, resulting in the superior mechanical properties of regenerated silk fibers. When taken together, the overall data tend to provide evidence that the precise nature of the primitive conformations of the fibroin solutions in hexafluoro solvents, i.e., the preferred 3_{10}-helical structure in HFIP and essentially disrupted α-helical structure in HFA, under relevant artificial spinning process exhibit a defined and distinctive influence on the structural as well as mechanical properties of the regenerated silk fibers.

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