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Characterization of water in hydrated *Bombyx mori* silk fibroin fiber and films by ^2H NMR relaxation and ^{13}C solid state NMR

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Abstract

The mechanical properties of *Bombyx mori* silk fibroin (SF), such as elasticity and tensile strength, change remarkably upon hydration. However, the microscopic interaction with water is not currently well understood on a molecular level. In this work, the dynamics of water molecules interacting with SF was studied by ^2H solution NMR relaxation and exchange measurements. Additionally, the conformations of hydrated $[3-^{13}\text{C}]\text{Ala-}$, $[3-^{13}\text{C}]\text{Ser-}$ and $[3-^{13}\text{C}]\text{Tyr-SF}$ fibers and films were investigated by ^{13}C DD/MAS NMR. Using an inverse Laplace transform algorithm, we were able to identify four distinct components in the relaxation times for water in SF fiber. Namely, A: bulk water outside the fiber, B: water molecules trapped weakly on the surface of the fiber, C: bound water molecules located in the inner surface of the fiber and D: bound water molecules located in the inner part of the fiber were distinguishable. In addition, four components were also observed for water in the SF film immersed in methanol for 30 seconds, while only two components for the film immersed in methanol for 24 hours. The effects of hydration on the conformation of Ser and Tyr residues in the site-specific crystalline and non-crystalline domains of ^{13}C selectively labeled SF, respectively, could be determined independently. Our measurements provide new insight relating the characteristics of water and the hydration structure of silk, which are relevant in light of current interest in the design of novel silk-based biomaterials.

Graphical Abstract

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The mechanical properties of *Bombyx mori* silk fibroin (SF) change remarkably upon hydration. However, the microscopic interaction between SF and water is not currently well understood on a molecular level. We were able to identify four distinct components in the relaxation times for water in SF fiber by ^2H solution NMR relaxation and exchange measurements. In addition, the effects of hydration on the conformation of Ser and Tyr residues in the site-specific crystalline and non-crystalline domains of ^{13}C selectively labeled SF, respectively, could be determined independently. Thus, our measurements provide new insight relating the characteristics of water and the hydration structure of silk, which are relevant in light of current interest in the design of novel silk-based biomaterials.

Keywords

Bombyx mori silk fibroin; fiber; film; ^2H NMR relaxation; ^{13}C DD/MAS NMR

1. Introduction

Bombyx mori silk fibroin (SF) has been used as a biomaterial because of its excellent mechanical properties and biocompatibility [1]. The mechanical properties of SF fibers, such as elasticity and tensile strength, change distinctly and reversibly as a function of the water content. For example, the elasticity increases with increasing water content at the expense of the rupture strength [2]. Because these biomaterials are usually used in a hydrated state, it has been of long-standing interest to quantify the interaction between water and SF. Several techniques such as Raman, IR, near-IR, XRD, and DSC etc have been used to understand the effect of hydration on SF [3–5]. Generally, water in the SF-water system is divided into three categories: free water, freezing bound water, and non-freezing bound water which predominates in the plasticizing effect on the SF chains [3]. One might speculate that the hydration of SF may arise from water molecules penetrating into the relatively disordered domains, such as the non-crystalline domain, and increase chain motion due to disruption of the hydrogen bonding in these regions by the strong interaction of water molecules with polar groups of the SF chain. However, the hydration mechanism of SF, including the microscopic interactions with water, is still not completely understood.

Compared with the spectroscopic methods mentioned above, NMR may clarify nano-structural features in the hydrated SF. Site-specific information of local structure may be obtained by ^{13}C selectively labeled SF [6–13]. One may readily prepare labeled $[3-^{13}\text{C}]\text{Ser-}$, $[3-^{13}\text{C}]\text{Tyr-}$ and $[3-^{13}\text{C}]\text{Ala-SF}$ by rearing silkworms with an artificial diet containing the ^{13}C labeled amino acids $[3-^{13}\text{C}]\text{Ser}$ and $[3-^{13}\text{C}]\text{Tyr}$. From the reported primary structure of SF, Ser residues are present predominantly in the crystalline domains, Tyr residues

predominantly in the non-crystalline domains, and Ala residues in both domains [13–15] (see Supplementary data s-1). Additionally, in the ^{13}C NMR spectra of SF in both solution and solid states, the ^{13}C chemical shifts of individual amino acid residues exhibit strong conformation-dependence, and are therefore very useful for studying local conformations[10]. Thus, ^{13}C NMR measurements with these ^{13}C selectively labeled SF samples are expected to provide local conformation of specific amino acids in crystalline and non-crystalline domains separately, which allows for details of the nano-structural features of SF in the wet state. In addition, by hydration, the mobility of the amino acid residues will increase partially and therefore one may monitor changes in both mobile and immobile states quantitatively. The ^{13}C Dipolar Decoupling (DD) /Magic Angle Spinning (MAS) NMR method allows for observing signals from both mobile and immobile domains. In recent work [15], ^{13}C DD/MAS NMR was used to determine the local conformation of $[3-^{13}\text{C}]\text{Ala-}$, $[3-^{13}\text{C}]\text{Ser-}$, and $[3-^{13}\text{C}]\text{Tyr-}$ *B. mori* SF fiber in a hydrated state. This study showed that ^{13}C solid state NMR coupled with ^{13}C selective labeling and peak deconvolution analysis is a very powerful method for analyzing the local structure of SF and associated structural changes induced by hydration. SF has been used as a biomaterial in several forms (e.g. fiber, mat, film, hydrogel, and sponge)[1]. It is expected that the degree of hydration varies between the fiber and film, for example, reflecting differences in the β -sheet fraction and morphology, surface area of SF interacting with water, amount of bound water, and so on. Thus, it is of interest to compare the structure and interaction with water molecules between the hydrated fiber and film to design novel silk-based materials.

NMR relaxation has also proven to be useful for monitoring the dynamics and distribution of water molecules interacting with SF. ^1H pulsed NMR was used to study the dynamics of water molecules absorbed in SF fibers, films, and powders [16]. Magnetization of dry SF by the solid-echo method showed a single Gaussian decay, while two components were observed in the solid-echo signals of films containing 6–10 w/w% water. An immobile component with a spin-spin relaxation time (T_2) value of 11 μs was attributed to the SF, and the mobile component with T_2 with relaxation time ranging from 50 to 200 μs , (depending on the SF sample) was attributed to bound water. Rodin and Knight [17] studied dynamics of water in *B. mori* SF fibers by using ^1H DQF NMR. The ^1H DQF NMR signal of water in SF fibers with low water content (6.5 w/w %), prepared by keeping samples in a desiccator in a saturated water vapor atmosphere, was observed. These provided insight into the local ordering and anisotropic motion of water molecules that give rise to non-vanishing ^1H - ^1H dipole interactions. At higher water content, 18 w/w%, the ^1H DQF NMR was not observed, indicating more rapid motion of water.

Recently, various two-dimensional (2D) relaxation and diffusion correlation and exchange NMR experiments based on an inverse Laplace transform (ILT) were proposed. These methods allow for studying the distribution of water compartments, based on their dynamical characteristics, and mutual exchange in porous media such as polymers and biological tissues [18–27]. This approach has been applied to study the dynamics and distribution of water in hydrated elastin and spider silk fibers [28–30]. Using an ILT algorithm, four components of waters with different relaxation times in hydrated fibers were observed in ^2H T_1 - T_2 correlation and T_2 - T_2 exchange experiments. For ^2H nuclei, the relaxation mechanism is dominated by the quadrupolar interaction, which is greater than that

of ^2H - ^1H , ^1H - ^1H , and ^2H - ^2H dipolar interactions, and a more sensitive probe of molecular motion of water molecule in silk samples [31,32]. Therefore, this approach may be used to probe the dynamics and interaction of water molecules in the hydrated SF systems.

In this paper, the dynamics of water molecules interacting with SF (fiber and films) was studied by ^2H T_1 - T_2 and T_2 - T_2 NMR methods. SF films for use as a biomaterial are usually prepared from the aqueous solution as cast film, but this film is water-soluble. Alteration of the SF structure was induced by treatment with methanol for varying times and water-insoluble films with different structure were obtained [16, 33, 34]. In this study, the SF fiber and film samples were immersed in $^2\text{H}_2\text{O}$ for more than one day in an NMR sample tube and then ^2H relaxation of water was observed with the samples immersed in $^2\text{H}_2\text{O}$. Therefore, our NMR observation is sensitive to fast motion and exchange with the water molecules interacting with the SF sample, which is different from the dynamics of water molecules acting as a plasticizer in the SF sample as mentioned above [3–5, 16]. The conformation of hydrated $[3\text{-}^{13}\text{C}]\text{Ala}$ -, $[3\text{-}^{13}\text{C}]\text{Ser}$ - and $[3\text{-}^{13}\text{C}]\text{Tyr}$ -SF films was determined for Ala, Ser and Tyr residues independently with ^{13}C DD/MAS NMR, and compared with those obtained for the hydrated SF fiber. As detailed in the Materials and methods section below, the hydrated SF samples were removed from water following immersion for one night prior to ^{13}C DD/MAS NMR measurements. On the other hand, our ^2H relaxation studies involved having samples immersed in excess bulk of $^2\text{H}_2\text{O}$ during NMR experiments.

2. Materials and methods

2.1 Preparation of ^{13}C labeled SF Fibers and Films

The ^{13}C selective labeling of both Ser C β and Tyr C β carbons of SF was performed by oral administration of an artificial diet with ^{13}C -labeled amino acids, both $[3\text{-}^{13}\text{C}]\text{Ser}$ and $[3\text{-}^{13}\text{C}]\text{Tyr}$ to the larvae. The ^{13}C labeling of Ala C β carbon was performed by transamination from $[3\text{-}^{13}\text{C}]\text{Ser}$ in the silkworm [11]. The ^{13}C labeling sites of these amino acids are shown in Supplementary data s-2. For preparation of SF films, the degummed ^{13}C -labeled SF fibers were dissolved in solvent system of $\text{CaCl}_2/\text{H}_2\text{O}/\text{EtOH}$ (mole ratio: 1/2/1) for 1 hr at 70 °C to obtain 10 w/v% SF aqueous solution and then dialyzed against distilled water for 3 days at 4 °C using a cellulose membrane (MWCO 14,000). After dialysis against pure water, approximately 5 w/v% SF solution was obtained and centrifuged 30 min at 4 °C under 18,000 rpm to remove impurities. The supernatant SF solution was poured to plastic plates and then dried to obtain the SF films [16, 33, 34]. The water-soluble films (thickness ranging from 110–140 μm) were immersed in 80% methanol aqueous solution for 30 s or 24 hrs for insolubilization. The SF films immersed in 80% methanol for 30 s or 24 hrs are denoted as 30 s MeOH film and 24 hrs MeOH film, respectively. The difference in the detailed molecular structure of the SF films between 30 s MeOH and 24 hrs MeOH treatments was studied using spin-label ESR, ^{13}C solution NMR and FT-IR in detail in our previous paper [33].

The degrees of swelling of the SF samples were determined according to the following equation.

$$\text{Degree of swelling (wt \%)} = ((W_{\text{wet}} - W_{\text{dry}}) / W_{\text{dry}}) \times 100$$

where W_{wet} is the weight of the hydrated SF sample and W_{dry} is the weight of the dried SF sample. In the preparation of the hydrated SF samples, they were immersed in H_2O for at least one day. This duration is sufficient to obtain a fully hydrated SF sample [33, 34]. The weight of the hydrated SF sample was determined after wiping excess water off the samples. On the other hand, the weight of the dried SF sample was determined after setting the samples in 60°C oven more than 3 days. The degree of swelling was 5, 30 and 103 wt% for SF fiber, 24 hrs MeOH and 30 s MeOH films, respectively. These hydrated SF films were used for the ^{13}C DD/MAS NMR experiment after being cut to insert into a 5 mm outer diameter NMR tube. On the other hand, for 2D ^2H T_1 - T_2 and T_2 - T_2 correlation experiments, the SF samples were immersed in $^2\text{H}_2\text{O}$ more than one day in a 5 mm NMR sample tube and then ^2H NMR relaxation of water was observed with the SF samples immersed in excess bulk of $^2\text{H}_2\text{O}$.

2.2 ^{13}C DD/MAS NMR Observation

The ^{13}C DD/MAS NMR spectra of the hydrated SF films were observed using a Bruker Avance 400 NMR spectrometer. A specially designed commercially available NMR rotor was used to observe the spectra to prevent dehydration of the hydrated samples. Details of the NMR conditions for the DD/MAS NMR experiments are already described in a previous paper in detail [15]. The number of scans was 8,192 for ^{13}C DD/MAS NMR observations with TPPM ^1H decoupling. Experiments were performed using a MAS rate of 8 kHz, a recycle delay of 10 s, and ^1H 90° pulse of 3.1 μs . No line broadening was applied for data processing. The rotor size used in this study had a 4 mm outer diameter, and approximately 30 mg sample was used.

2.3 Deconvolution Analysis of ^{13}C DD/MAS NMR Spectra

The Ala C β peak in the ^{13}C DD/MAS NMR spectra of the hydrated SF fiber and films was assigned to the Ala C β carbon (16.5 ppm : random coil with fast and slow chain dynamics, 19.6 ppm: β -sheet A, and 21.7 ppm: β -sheet B). Similarly, the Ser C β peak in the ^{13}C DD/MAS NMR spectra was assigned to the Ser C β carbon (61.5 ppm : random coil with fast and slow chain dynamics, 64.0 ppm: β -sheet A, and 65.5 ppm: β -sheet B). Details of these assignments have been published in refs.11 (Ser) and 12 (Ala), respectively. For the Tyr C β peak, the peak of 36.1 ppm was assigned to a random coil with fast and slow chain dynamics, and the 40.3 ppm peak to β -sheet, respectively although the lower field peak overlapped with the natural abundance Gly C α peak at 42.6 ppm. All the deconvolution analyses were performed by assuming a Gaussian lineshape [15].

2.4 2D ^2H T_1 - T_2 Correlation Experiments

All of the ^2H relaxation experiments were performed on a Varian Unity 200 MHz NMR spectrometer with a liquid state NMR probe without spinning. The variable temperature T_1 - T_2 experiments were conducted at 15° , 20° , 25° , 30° , 40° and 50°C for the SF fiber. Similarly, temperature dependent T_1 - T_2 experiments were performed for 30 s MeOH and 24

hrs MeOH films. Pulse sequences used for the T_1 - T_2 experiments are shown in Fig. 1(top); the magnetization is inverted by the initial 180° pulse (52 μ s) and then recovers to thermal equilibrium during the variable delay t_1 by relaxation time T_1 . The 90° pulse (27 μ s) following the delay τ rotates the magnetization into the transverse plane followed by the 180° pulses and the NMR echo signal is stroboscopically detected at $2n\tau$ time with a Carr-Purcell-Meiboom-Gill (CPMG) pulse train with τ set to 0.350 ms [20]. The number of scans accumulated in these experiments was 24 and a recycle delay of 15 s was used between experiments. The CPMG (π pulse train) is used to observe T_2 relaxation decay due to the quadrupolar interaction by removing field inhomogeneity and chemical shift interactions (any Hamiltonians that scale as a constant multiplied by I_z). All measurements of the relaxation times T_1 , T_2 by this method are made in the presence of possible exchange and should therefore be interpreted as the apparent relaxation times.

2.5 Deuterium $2D$ 2H T_2 - T_2 Correlation Experiments

For the T_2 - T_2 measurements, two CPMG sequences are used separated by an experimentally controlled mixing time; exchange between reservoirs corresponding to the ensemble of water molecules in the compartment, is monitored by the observation of cross peaks in the resulting two-dimensional inverse Laplace transformation. All T_2 - T_2 measurements were performed at 25°C . The pulse sequence used for the T_2 - T_2 correlation experiment is shown in Fig. 1(bottom). Similar to the T_1 - T_2 experiments, an inter-pulse spacing of $\tau = 0.350$ ms was used with the same pulse durations, and 24 scans were accumulated [30]. The number of loops used in our study was $m = 89$ (logarithmically spaced) and $n = 6,000$ (transverse axis). By incrementing the mixing time (t_m), a series of T_2 - T_2 correlation maps were obtained, and exchange of water between different compartments in SF fiber was monitored via the time dependence of the cross peak intensities. The average experimental time was approximately 15 hours per experiment, due to necessary delays to prevent sample heating and dehydration. Here, the cross peaks in T_2 - T_2 correlation experiment are observed in the presence of exchange, and are absent without exchange [35, 36]. In all of the relaxation measurements, we implemented the BRD method [37] for obtaining the optimal regularization parameter [19]. In either the T_1 - T_2 or the T_2 - T_2 exchange measurements we found that the signal intensities and relaxation times did not change significantly with the regularization parameter, although the resolution was observed to reduce. In a previous work [29], we highlight the efficacy of the algorithm used for simulated data (where 5 different reservoirs of known relaxation times and signal intensities were simulated), and found that the ILT results were largely signal to noise dependent. In all measurements presented in this study we reproduced on 2 different samples and replicated across 2 to 3 measurements.

3. Results and Discussion

3.1 ^{13}C DD/MAS NMR Spectra of SF

The ^{13}C - ^1H cross polarization (CP) /MAS NMR method has been usually used for the structural analysis of SF [10,11, 38]. However, the presence of water causes an increase in chain mobility and consequential loss in CP signal of the amino acid residues in the SF that are hydrated by water [7, 8]. Therefore, we observed ^{13}C DD /MAS NMR spectra to detect the mobile domains as well as the immobile domains of SF quantitatively [15].

Fig. 2 shows the ^{13}C DD/MAS NMR spectra of $[3-^{13}\text{C}]\text{Ala-}$, $[3-^{13}\text{C}]\text{Ser-}$ and $[3-^{13}\text{C}]\text{Tyr-SF}$ fiber in a hydrated state published recently [15], and those of 30 s MeOH and 24 hrs MeOH films of $[3-^{13}\text{C}]\text{Ala-}$, $[3-^{13}\text{C}]\text{Ser-}$ and $[3-^{13}\text{C}]\text{Tyr-SF}$ in a hydrated state. The peak at approximately 110 ppm comes from spacer signals of the rotor and not from the samples studied. Both broad and sharp peaks were observed for individual carbon signals of SF, which correspond to unhydrated carbons with restricted motion and hydrated carbons with higher mobility, respectively. The fraction of the sharp peak in each carbon increases in the order of SF fiber, 24 hrs MeOH film, and 30 s MeOH film. The ^{13}C labeling of the Tyr $\text{C}\beta$ and Ser $\text{C}\beta$ carbons of SF, as described in the sample preparation section, allows for conformational studies of these residues. It was noted that the intensity of Ala $\text{C}\beta$ peak increases due to transamination from $[3-^{13}\text{C}]\text{Ser}$ in the silkworm. Interestingly, it is possible to obtain domain-specific structural information from these NMR spectra, because Ser residues are present predominantly in the crystalline domain, and Tyr predominantly in the noncrystalline domain, while Ala residues are abundant in both domains [14] (Supplementary data s-1). Therefore, the effects of hydration on site-specific crystalline and noncrystalline domains of SF may be obtained. The fraction of local conformation of Ser, Tyr and Ala residues of SF in a hydrated state was determined from peak deconvolution analysis as described in the following section.

3.2 Determination of the Fraction of Local Conformation by Deconvolution Analysis

After hydration, sharp peaks appear in the spectra indicating random coil chemical shifts. These peaks were observed for Ala, Ser and Tyr ^{13}C labeled $\text{C}\beta$ carbons as summarized in Fig. 3, and are marked by blue, or light yellow (broad peak) [15]. As reported previously,¹² the Ala $\text{C}\beta$ peak in the ^{13}C CP/MAS NMR spectrum of SF reflects the inhomogeneity of the SF sample. The Ala $\text{C}\beta$ peaks from the insoluble crystalline fraction after chymotrypsin cleavage consists of three peaks: 16.5 ppm for distorted β -turn and/or random coil, 19.6 ppm for the β -sheet peak A, and 21.7 ppm for the β -sheet peak B. The difference between β -sheet peaks, A and B, arises from difference in the inter-molecular structure arrangement as discussed in detail elsewhere [12]. On the other hand, the Ala $\text{C}\beta$ peak of the noncrystalline domain obtained by subtracting the crystalline fraction peak from the whole peak consists of two peaks: 16.5 ppm for random coil, and 21.3 ppm for distorted β -sheet. In this paper, we assigned the Ala $\text{C}\beta$ peak 16.5 ppm to a random coil, the 19.6 ppm peak to β -sheet A, and 21.7 ppm peak to β -sheet B [15]. Similarly, the 61.5 ppm peak arises from a random coil, and the 64.0 ppm and 65.5 ppm peaks arise from β -sheet A and β -sheet B for the Ser residues, respectively as reported previously [11]. The assignment is relatively complicated for Tyr residues. The peak of 36.1 ppm arises from a random coil, and the 40.3 ppm peak is β -sheet for the Tyr residues, respectively. However, the lower field peak overlaps with natural abundance Gly $\text{C}\alpha$ peak at 42.6ppm [10]. The ^{13}C DD/MAS NMR spectrum changes remarkably at the random coil region of Ala and Ser residues while there is no significant change at the β -sheet region, as reported previously [6, 15]. Kishore et al. showed that hydration increases the molecular mobility of spider silk significantly by solid state NMR relaxation studies [39]. Holland et. al. observed two Ala $\text{C}\beta$ peaks in the ^{13}C DD/MAS NMR spectra of spider silks by hydration, which are assigned to β -sheet (broader peak at lower field) and random coil (sharper peak at higher field), respectively [8,10]. The character of the hydrated SF spectra is the same as those reported by them, and we further

attempted to determine the fraction of each conformation in the hydrated state quantitatively by deconvolution analysis, described below.

The fraction of β -sheet and two random coil distributions with fast and slow chain dynamics was determined from the peak area, by assuming Gaussian lineshapes [15], as shown in Fig. 3. The results from this analysis are summarized in Table 1. For the SF fiber, the fraction of β -sheet structure decreases in the order of Ala, Ser and Tyr, while the fractions of hydrated random coil structures decrease in the order of Ser, Ala and Tyr. Until now, it has been postulated that the hydration of SF may involve water molecules penetrating into the relatively disordered domains, which may include noncrystalline domains, and result in an increase in chain motion due to disruption of hydrogen bonds by the strong interaction of water molecules with polar groups of the SF chain [3, 40]. The results reported in this work indicate that Ser rich domains are the most water-accessible due to the presence of OH group in the side chain. Of course, the Ser residues in β -sheet formation are expected not to allow for easy access to water molecules. However, half of the Ser residues exhibit a random coil structure, and a fraction of Ser residues (11%) in random coil structure appeared to allow access to water molecules (Table 1). On the other hand, although there are also OH groups in the side chains of Tyr residues located in the noncrystalline domains, the presence of the aromatic groups may induce a hydrophobic environment that may block the hydration of these residues in the SF fiber [41]. Thus, the difference in the hydrophilic and hydrophobic characteristics between Ser and Tyr residues seems significant for water penetration, rather than the difference in the position that is present in crystalline and noncrystalline domains.

In the preparation of the SF films, the degummed SF fibers were dissolved in $\text{CaCl}_2/\text{H}_2\text{O}/\text{EtOH}$ solvent system and then the solution was dialyzed against water. The β -sheet structures in the SF fiber destroy completely and convert to random coil. The SF films were prepared after drying the aqueous solution of SF and then immersed in methanol for insolubilization [16, 33, 34]. During this methanol treatment process, the β -sheet structure of SF film rebuilds again, but the inter-molecular arrangement of the β -sheet structure is different from the β -sheet in the SF fiber [42]. Thus the decrease in the fraction of the β -sheet structure in the crystalline domain, $(\text{Ala-Gly-Ser-Gly-Ala-Gly})_n$ [14] compared with the fraction of SF fiber occurs. This is the reason why the β -sheet fraction of Ala and Ser residues in SF films decreases compared with SF fiber although no significant changes of the β -sheet fraction were observed for Tyr residues. By hydration, the increase in the fraction of hydrated random coil form with high mobility is more pronounced for Ala and Ser residues, especially for 30 s MeOH film. This means that the random coil form of 30 s MeOH film is relatively easy to access with water molecules than the 24 hrs MeOH film which is due to incomplete β -sheet formation of the SF film surface by methanol because of short immersion time 30 s. In our previous paper [33], we prepared 8 kinds of the SF films by changing the times immersed in methanol from 30 s to 48 hrs (30s, 1 min, 3min, 10min, 1hr, 8hrs, 24hrs and 48hrs). Then, the degree of swelling of the SF films in H_2O was determined. The degree of swelling of each SF film determined according to the method described in Materials and methods increases rapidly with increasing the immersion time in H_2O and reaches the equilibrium value within one day. This tendency was independent of the films prepared by changing the methanol treatment time. The equilibrium value of the

SF films decreases remarkably from 30s methanol treatment to 3 min treatment. At more than 3 min treatment, however, the value decreases slightly. Thus, increase in the fraction of the hydrated random coil and decrease in the fraction of the unhydrated random coil of Ala, Ser and Tyr residues occurs for 30 s MeOH film compared with 24 hrs MeOH film [33].

3.3 ^2H T_1 - T_2 Observation of $^2\text{H}_2\text{O}$ in SF- $^2\text{H}_2\text{O}$ Fiber

It is of interest to obtain further information on the water-SF interaction from the viewpoint of the dynamics and distribution of water. Recently, 2D correlation methods, based on an ILT, have been implemented to study mobile water molecules in multi-compartment systems [18–30]. Fig. 4 shows the resulting 2D ILT from a ^2H T_1 - T_2 correlation diagram of the SF fiber in the range of $10^{-2} \text{ s} < T_1 < 1 \text{ s}$ and $10^{-3} \text{ s} < T_2 < 1 \text{ s}$ at 25 °C. For these experiments, the SF fiber was immersed in $^2\text{H}_2\text{O}$ for more than one day in NMR sample tube, and then ^2H T_1 - T_2 correlation experiments were performed. The T_1 - T_2 correlation data in Fig. 4 revealed the presence of four distinct water reservoirs; three water reservoirs, B–D, together with surrounding bulk water reservoir, A. Most of the ^2H signal arises from bulk $^2\text{H}_2\text{O}$ outside of the fibers. Details of the T_1 and T_2 values with the experimental errors together with the relative signal intensities are listed as a function of observed temperature are provided in Supplementary data s-3. The fraction of the reservoirs denoted A, B, C and D was estimated to be 77%, 21%, 2 % and 0.004% respectively. The reservoirs, B, C and D are characterized by an ensemble of water molecules exhibiting different dynamical characteristics over the time scale of the NMR measurement spanning 0.006 – 0.44 s and 0.22–0.47 s in the T_2 and T_1 domains, respectively. Thus, T_2 is more sensitive to exchange and dynamical processes in the NMR relaxation times than T_1 . The dominant reservoir A, arises from bulk water outside the fibers exhibiting highly isotropic fast motion as $T_1 \approx T_2$. In contrast, T_2 values were significantly shorter than T_1 values for reservoirs, B–D. The remaining three water reservoirs, B–D, may be assigned to exchangeable water molecules interacting with the SF fiber in three different degrees of binding interactions.

Rodin and Knight [17] studied dynamics of water in *B. mori* SF fibers by using ^1H DQF NMR. In this study, we investigated the dynamics of ^2H nuclei. For ^2H nuclei, the relaxation mechanism is dominated by fluctuation of the quadrupolar interaction, which is greater than that of ^1H - ^1H , ^2H - ^2H , and ^2H - ^1H dipolar interactions, and a more sensitive probe of molecular motion [31, 32, 43]. These 2D ^2H relaxation data thus provide more detailed information than previous ^1H DQF NMR measurements regarding the distribution and dynamics of water reservoirs in the water-SF system [17]. The reservoir denoted B in Fig. 4 may be assigned to water molecules trapped weakly on the surface of the SF fibers. This was verified by wiping the surface of the SF fibers, and removing any bulk water from the NMR tube (discussed in detail in section 3.5). The reservoir C can be assigned to bound water molecules located in the inner surface of the SF fiber. In addition, the reservoir denoted D reflects the bound water molecules located in the inner part of the SF fiber. The presence of such exchangeable water molecules with relatively restricted motion was already reported in studies of water-hydrated elastin, spider silk, and a perfluorinated ionomer system by 2D relaxation, diffusion, and exchange NMR experiments based on inverse Laplace transform methods [27, 29, 30]. Shen et al. reported microstructure of SF fiber by transmission electron microscopy, selected area electron diffraction, wide-angle X-ray scattering and low-

voltage high-resolution scanning electron microscopy. The crazes were observed along the fiber axis in the degummed SF fiber where all the crazes ran parallel to fiber axis [44]. Using ^1H NMR cryoporometry, Zhu et al. determined the pore size distribution of fresh SF fiber, which was strongly asymmetric with a well-defined maximum pore size at 1.1 nm [45]. Thus, heterogeneous microstructure including voids was observed inner parts of the SF fiber, which may give rise to the appearance of several peaks in the 2D T_1 - T_2 map. Lastly, one may argue that the peak D arises from exchangeable hydroxyls which were partially converted into deuterioxylys [46]. Below, we show that the exchange between reservoirs D and C, B, or A is longer than 8 ms.. This observation would indicate that ^2H nuclei assigned to reservoir D arise from $^2\text{H}_2\text{O}$ and not deuterioxylys.

The temperature dependent relaxation times of the four reservoirs in SF fiber- $^2\text{H}_2\text{O}$ system were also measured. Fig. 5 summarizes the plots of the T_1 and T_2 values of A to D against temperature, and the T_1 and T_2 values are listed as a function of temperature in Supplementary data s-3. The T_1 and T_2 values of the dominant reservoir A increase linearly with temperature and are characterized by $T_1 \approx T_2$, indicating that the correlation time is extremely short and indicative of isotropic fast motion. The ^2H T_1 values of reservoir B also increase with increasing temperature, but the degree of the increase is slightly smaller compared to A. The interesting point is the case of the ^2H T_2 of B - there was no significant increase with increasing temperature, and also considerably smaller values than the T_2 value of A are observed. It is noted that T_2 values for this reservoir were significantly smaller than T_1 - this result may indicate that water molecules in the reservoir may be exchanging with less mobile water molecules due to interaction with SF, and diffusing to different reservoirs, which subsequently alters the observed relaxation times. Although we did not measure the temperature dependence of this exchange (measurements were performed only at 25 °C, discussed below), the observation of a reduction in the T_2 would indicate either a reduction in tumbling motion of the deuterium nucleus and/or a reduction of exchange between reservoir B and reservoir A, or an increase in exchange between B and reservoirs with less mobile state. Almost no change in T_1 and slight decrease in T_2 were observed for reservoirs C and D over the temperature range of 15 to 50 °C. For further quantitative discussion, a diffusion experiment may shed light on the molecular mobility of water, the temperature dependent tortuosity and permeability of the silk fiber, as the transverse relaxation process is sensitive to the slower molecular motions present in the local confinement compared to the longitudinal relaxation.

3.4 ^2H T_2 - T_2 exchange measurements of $^2\text{H}_2\text{O}$ in SF- $^2\text{H}_2\text{O}$ Fiber

In order to obtain direct evidence of the exchange among these reservoirs, a ^2H T_2 - T_2 exchange measurement was performed. Fig. 6 highlights the resulting T_2 - T_2 exchange measurements of the SF fiber- $^2\text{H}_2\text{O}$ system at 25 °C for mixing times (a) 2.5 ms, (b) 8ms, (c) 64ms and (d) 256 ms, respectively [30]. While exchange takes place throughout the T_2 - T_2 2D exchange measurement, these times denote the delay between two consecutive CPMG sequences. The 2D T_2 - T_2 correlation results indicated that the four reservoirs do not exchange with one another over a time scale < 8ms as no cross peaks were observed. However, for longer time scales (e.g. 64 and 256 ms) we observe exchange between the various reservoirs. Note that the water reservoir denoted D disappears in the 64ms and

256ms T_2 - T_2 exchange measurement, as the peak is attenuated by T_2 relaxation during the CPMG cycles. In principle many T_2 - T_2 2D exchange measurement, with varying exchange times, are required to accurately measure the exchange rates and are time consuming due to necessary delays to prevent sample dehydration and heating. Here, experiments were performed to merely provide evidence of the signature of exchange between the observable sites.

3.5 Effect of removing bulk water on the ^2H T_1 - T_2 correlation maps of SF- $^2\text{H}_2\text{O}$ Fiber

In order to demonstrate the effect removing the bulk water from the samples, we obtained the 2D ^2H T_1 - T_2 ILT correlation maps of $^2\text{H}_2\text{O}$ in hydrated SF fiber before and after blotting the samples with a kimwipe. The results obtained from this process are shown in Fig. 7a) and b), respectively, together with Table 2. It is impossible to completely remove all of the bulk and surface water as some water will be inevitably squeezed out of the sample while handling and placing it in the NMR tube. Thus, some bulk water was present on the surface of the sample, as well as on the inner surface of the NMR tube. However, we note that the signal intensity in Fig. 7a) and b) is on a logarithmic scale and that both reservoirs A and B are observed to reduce in intensity by 2 to 3 orders of magnitude (Table 2). This indicates that water molecules in reservoirs A and B are easily removed from the fibril. The absolute signal intensity of peak A decreased from 26811 to 1029 and that of peak B decreased from 19844 to 3275, after blotting the samples with a kimwipe. This result indicates that peak B showed slightly stronger interaction with the surface of fibril as compared to the peak A although both the signal intensities of reservoirs A and B are suppressed significantly. It is also noted that absolute signal intensity of peak C reduced from 2706 to 1320. This indicates that water molecules in reservoir C interact more strongly with the surface of fibril than reservoirs A and B do. Furthermore, the absolute peak intensity of peak D reduced from 353 to 342. It is of interest to note that the signal corresponding to reservoir D did not reduce in the blotting process, which removed much of the bulk water. This clearly indicates that reservoir D is located deep within the fibril and is well-isolated from the other reservoirs. The T_2 value of reservoir D is much smaller in comparison to other reservoirs, indicating that the motion of water molecules in this reservoir is more restricted in comparison to that of molecules in reservoirs C, B, or A. It is also noted that the T_1/T_2 ratios of reservoirs A to D changed only slightly by removal of bulk water, indicating that the mobility of water in each reservoir is not altered by the blotting process.

3.6 ^2H NMR relaxation of $^2\text{H}_2\text{O}$ in SF- $^2\text{H}_2\text{O}$ Films

Fig. 8 shows the resulting 2D ILT from a $^2\text{H}_2$ T_1 - T_2 correlation measurement of the 30 s MeOH (a) and 24 hrs (b) SF films- $^2\text{H}_2\text{O}$ system. Details of the T_1 and T_2 values with the experimental errors together with the fractions are listed as a function of observed temperature in Supplementary data s-4. In the preparation of the SF films, the SF fibers were dissolved in $\text{CaCl}_2/\text{H}_2\text{O}/\text{EtOH}$ solvent system. Thus, the β -sheet structures in the SF fiber including heterogeneous microstructure such as voids destroy completely. However, the 30 s MeOH film-water system reveals three reservoirs except for free bulk water, which was also observed in the SF fiber. As mentioned above, the random coil form of 30 s MeOH film is relatively easy to access with water molecules than the 24 hrs MeOH film which is due to incomplete β -sheet formation of the SF film surface by methanol because of short

immersion time 30 s. Thus the observation of three reservoirs except for free bulk water, in a $^2\text{H}_2$ T_1 - T_2 correlation measurement seems related with the structure of the 30 s MeOH film. The reservoir (two points) denoted B in Fig. 8 (a) is assigned to the water molecules trapped weakly on the film surfaces; reservoir B in SF film arises from water molecules trapped in the surface of the SF film, as mentioned in the previous section. The T_1 and T_2 values of reservoir B are in the same order between SF fiber and 30 s MeOH film. However, the fractions are B (34 %), C (23 %) and D (43 %), respectively, for 30 s MeOH film if the fraction without the reservoir A is normalized to be 100%, which are considerably different from the fraction of the reservoirs B (89 %), C (9 %), and D (2 %), respectively, of SF fiber at 25 °C. Namely, the fractions of the latter two reservoirs, C and D increase considerably in comparison to the SF fiber. These alterations appear to be related to the increased swelling for 30 s MeOH film compared with the fiber. In addition, we observed a marked difference between the SF fiber (Fig. 4) and 30 s MeOH film for the reservoirs C and D; both T_1 and T_2 values decrease considerably for the latter film.

The temperature dependence of these four reservoirs in SF film- $^2\text{H}_2\text{O}$ system is shown in Fig. 9. The T_1 and T_2 values of dominant reservoir A increase with temperature linearly exhibiting the same tendency as observed in the hydrated SF fiber. This observation indicates that bulk or surrounding water molecules are not bound to the SF films and exhibit isotropic fast motion. The ^2H T_1 value of reservoir B increases with increasing temperature up to 45 °C, but reduces slightly at 55 °C. On the other hand, with increasing temperature, the ^2H T_2 values of reservoir B tend to flatten up to 35 °C, and then decrease. For reservoir C, the T_1 value tends to flatten or slightly increase with increasing temperature, but the change in the T_2 values are more complex and there are multiple reservoirs at some temperatures. On the other hand, there was a temperature dependent trend for reservoir D. Namely, T_1 increases slightly and T_2 remains nearly constant with increasing temperature. These results might indicate that the water molecules are more strongly bound to the SF films than the SF fiber. Reservoirs B and C observed in the 30 s MeOH film disappeared in the 24 hrs MeOH film as shown in Fig. 8 (b) and only the reservoir denoted by * was observed for the 24 hrs MeOH film. Judging from the T_1 and T_2 values, the reservoir * is assigned as reservoir D. These measurements indicate that the 24 hrs MeOH film exhibits different inner and surface architecture than the 30 s MeOH film as mentioned above. The disappearance of water reservoir B may arise from more tightly formed surface architecture of the SF film due to immersion in methanol for 24 hrs, which would interfere with the penetration of water compared with 30 s MeOH film [33].

The absolute values of the T_1 and T_2 relaxation times of water molecules between the 24 hrs MeOH film and SF fiber reflect additional information worth noting. At 25 °C, for reservoir D, $T_1 = 0.22$ s and $T_2 = 0.0062$ s for the SF fiber, and $T_1 = 0.041$ s and $T_2 = 0.0033$ s for the 24 hrs MeOH film. For reservoir A, $T_1 = 0.47$ s and $T_2 = 0.44$ s for SF fiber, and $T_1 = 0.47$ s and $T_2 = 0.46$ s for the 24 hrs MeOH film. The temperature dependence of these two reservoirs in 24 hrs MeOH film- $^2\text{H}_2\text{O}$ system is shown in Fig. 10. T_1 remains nearly constant with increasing temperature, but T_2 increases slightly in lower temperature range for reservoir D. Thus, the degree of decrease of T_1 in D from A in the SF fiber is considerably smaller than that of T_1 in 24 hrs MeOH film although the change in T_2 is similar for the two samples. These results indicate that the water molecules in reservoir D

interacting with the SF fiber are located in an area which comprises the inner part of SF fiber, or in small pores in the fiber which exhibit strong binding interaction. On the other hand, water molecules in reservoir D in the 24 hrs MeOH film strongly interact with the SF film than the SF fiber and the mobility decreases considerably. The latter tendency is the same as the case of D at the 30 s MeOH film, as the relaxation times for this reservoir are similar to that at the 24 hrs MeOH film.

4. Conclusions

This work reports on a study of the hydration mechanism of *Bombyx mori* SF on a molecular level by two NMR methods. We implemented ^2H T_1 - T_2 and T_2 - T_2 NMR relaxation methods to probe the dynamics, distribution, and exchange of water molecules interacting with SF fiber. Using an inverse Laplace transform algorithm, four reservoirs with different relaxation times for the SF fibers are revealed. The duration of methanol treatment, 30 s vs 24 hrs, alters the distribution of water in SF films. The assignments are as follows. A: bulk water outside the SF, B: water molecules trapped weakly on the surface of SF, C: bound water molecules located inner surface of SF, and D: bound water molecules located in the inner part of SF. The two components in the ^1H NMR T_1 determination of hydrated SF film by water were already reported by us [47]. The merit of the ^2H T_1 - T_2 correlation images of SF fibers and films observed here is the observation of dynamics of water molecules in the SF materials in $^2\text{H}_2\text{O}$ without wiping the surfaces. This situation of the observation is extremely useful because such a biomaterial is generally set in the body fluids of animals. Secondly, we applied ^{13}C DD/MAS NMR to quantitatively determine the fraction of local conformations of Ser, Tyr and Ala residues of hydrated $[3-^{13}\text{C}]\text{Ala}$ -, $[3-^{13}\text{C}]\text{Ser}$ - and $[3-^{13}\text{C}]\text{Tyr}$ -SF fiber and films coupled with the peak simulations. It is noted that the difference in the hydrophilic and hydrophobic characters between Ser and Tyr residues may play a role rather than the difference in the position that is present in crystalline and non crystalline domains. Thus, these NMR analyses give new details relating to the states and dynamics of water together with the hydration structure of silk, which are relevant in light of current interest in the design of novel silk based biomaterials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Pulse sequences used for two-dimensional (top) T_1 - T_2 and (bottom) T_2 - T_2 relaxation experiments. In the top image, the phase cycling used for 2D T_1 - T_2 experiments was $\phi_1 = x, y, -x, -y$, $\phi_2 = x, x, -x, -x$, $\phi_3 = y, y, y, y$, and the receiver phase was $\phi_{\text{receiver}} = x, x, -x, -x$. The number of loops $n = 6000$. For 2D T_2 - T_2 experiments (bottom) the phase cycling used was $\phi_1 = x, -x, x, -x$, $\phi_2 = y, -y, y, -y$, $\phi_3 = x, x, -x, -x$, and the receiver phase was $\phi_{\text{receiver}} = x, -x, x, -x$.

Fig. 2.
 ^{13}C DD/MAS spectra of hydrated $[3-^{13}\text{C}]\text{Ala-}$, $[3-^{13}\text{C}]\text{Ser-}$ and $[3-^{13}\text{C}]\text{Tyr-}$ *B. mori* silk fibroin samples. (a) silk fiber, (b) 30 s MeOH treatment silk film and (c) 24 hrs MeOH treatment silk film. The peaks assigned to Ala C α , Gly C α and Ser C α and C=O carbons come from natural abundance contributions.

Fig. 3.

Observed and deconvoluted ^{13}C DD/MAS NMR spectra of Ala (a,b,c), Ser (d,e,f) and Tyr C β (g,h,i) carbons of hydrated $[3-^{13}\text{C}]\text{Ala-}$, $[3-^{13}\text{C}]\text{Ser-}$ and $[3-^{13}\text{C}]\text{Tyr-}$ *B. mori* silk fibroin samples. (a,d,e) silk fiber, (b,e,h) 30 s MeOH treatment silk film and (c,f,i) 24 hrs MeOH treatment silk film, respectively. The symbols shown in the spectra are defined as follows; ① β -sheet B, ② β -sheet A, ③ random coil, ④ hydrated random coil and ⑤ β -sheet.

Fig. 4. ^2H T_1 - T_2 ILT correlation map of $^2\text{H}_2\text{O}$ in hydrated SF fiber at 25°C . The assignments of reservoirs, A, B, C, and D are described in the text. The dotted line shown is a guide to the eye where $T_1 = T_2$.

Fig. 5. ^2H T_1 and T_2 of $^2\text{H}_2\text{O}$ in SF fiber - $^2\text{H}_2\text{O}$ system observed as a function of temperature. Shown are the relaxation-temperature dependences for reservoirs A through D, corresponding to the labeling used in Fig. 4. The T_1 and T_2 values, and T_1/T_2 ratios are summarized in Supplementary data s-3 together with the fractions.

Fig. 6. ^2H T_2 - T_2 exchange measurements of $^2\text{H}_2\text{O}$ in a SF fiber-water system at 25°C . The time between the CPMG trains used in the experiments are a) 2.5 ms, b) 8 ms, c) 64ms, and d) 256 ms. Cross peaks in these experiments are a signature of exchange between any two sites (e.g. in panel c) and d) exchange is observed between reservoirs C-B and B-A, see Fig. 4 for the definition of the various reservoirs s). As discussed in the text, peak D is missing in panels c) and d) as this reservoir of water undergoes T_2 decay during the first encoding stage and during the time between the two CPMG trains.

Fig. 7. ^2H T_1 - T_2 ILT correlation maps of $^2\text{H}_2\text{O}$ in hydrated SF fiber (a) before and (b) after blotting samples with a kimwipe at 13 °C. The assignments of reservoirs, A, B, C, and D are described in the text. The dotted line shown is a guide to the eye where $T_1 = T_2$. The T_1 and T_2 values, T_1/T_2 ratios and absolute signal intensities of reservoirs (A–D) are listed in Table 2.

Fig. 8. ^2H T_1 - T_2 ILT map of $^2\text{H}_2\text{O}$ in *B. mori* silk fibroin films–water systems at 25°C with (a) 30 s MeOH treatment and (b) 24 hrs MeOH treatment. The assignments of A, B, C and D in the left, and A and * in the right are described in the text. The dotted line shown is a guide to the eye where $T_1 = T_2$.

Fig. 9.

^2H T_1 and T_2 of $^2\text{H}_2\text{O}$ in 30 s MeOH SF films observed as a function of temperature. Shown are the relaxation-temperature dependences for reservoirs A through D, corresponding to the labeling used in Fig. 8. Measured relaxation times at some few temperatures (e.g. panel C, 15°C) show more than one measured value per given temperature. This is due to the distribution of peaks observed in the T_1 - T_2 ILT map that were grouped together (e.g. reservoirs B and C at some temperatures consisted of more than one spot). The T_1 and T_2 values, and T_1/T_2 ratio were summarized in Supplementary data s-4 together with the fractions.

Fig. 10.

^2H T_1 and T_2 of $^2\text{H}_2\text{O}$ in 24 hrs MeOH SF films observed as a function of temperature.

Shown are the relaxation temperature dependences for reservoirs A and D (* in Fig. 8 (b)).

The T_1 and T_2 values, and T_1/T_2 ratio are summarized in Supplementary data s-4 together with the fractions.

Table 1

Fraction (%) of different conformations from the ^{13}C DD/MAS NMR spectra of *B. mori* silk fibroin

Silk fibroin fiber (25 °C)				
Ala	β -sheet B	β -sheet A	random coil	hydrated random coil
	18	46	27	9
Ser	β -sheet B	β -sheet A	random coil	hydrated random coil
	17	33	39	11
Tyr	β -sheet B		random coil	hydrated random coil
	42		52	6
Silk fibroin film (25 °C)				
upper : 30 s MeOH treatment		lower : 24 hr MeOH treatment		
Ala	β -sheet B	β -sheet A	random coil	hydrated random coil
	14	34	31	21
	12	37	35	16
Ser	β -sheet B	β -sheet A	random coil	hydrated random coil
	11	28	41	20
	11	29	46	14
Tyr	β -sheet B		random coil	hydrated random coil
	41		49	10
	43		51	6

^2H T_1 and T_2 values, T_1/T_2 ratios and absolute signal intensities of reservoirs (A-D) in ^2H T_1 - T_2 correlation maps of $^2\text{H}_2\text{O}$ in SF fiber- $^2\text{H}_2\text{O}$ system before and after being blotted with a kimwipe (Fig. 7 (a) and (b), respectively). All experiments were performed at 13 °C. Note that the error bar shown in the T_1 and T_2 values indicates the spread in the resulting inverse Laplace transform map.

Table 2

Treatment	T_1	T_2	T_1/T_2	Absolute signal intensity
Before blotting	0.14 ± 0.01	0.0075 ± 0.0040	18.04	353
	0.23 ± 0.02	0.066 ± 0.010	3.43	2706
	0.31 ± 0.00	0.17 ± 0.06	1.79	19844
	0.38 ± 0.04	0.38 ± 0.07	1.00	26811
After blotting	0.13 ± 0.01	0.0065 ± 0.0040	19.88	349
	0.19 ± 0.01	0.057 ± 0.004	3.28	1320
	0.26 ± 0.01	0.12 ± 0.02	2.26	3275
	0.38 ± 0.03	0.38 ± 0.03	1.00	1029