NMR OF SILK FIBROIN 11. \( ^1H \) NMR ANALYSIS OF WATER ORIENTATION IN POROUS SILK FIBROIN MEMBRANE

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(Received July 4, 1988)

ABSTRACT

The physical state and orientation of the water trapped in porous Bombyx mori silk fibroin membranes were examined in the absence and presence of alkali metal salts, NaCl, KCl and LiCl by means of NMR spectroscopy. In spite of the high content of water (93%) in the membrane, doublet \( ^1H \) NMR peaks of the water molecules were observed and its separation depended strongly on the angle, \( \theta \), between the membrane surface and the magnetic field. This trend was independent of the presence of alkali metal ions in the water. From the relationship between the separation of the doublet and the angle, \( \theta \), the orientation of the proton-proton axis of the water molecule was found, on the average, parallel to the surface of the membrane. \( ^1H \) NMR peaks of the water and also, \( ^23Na \) NMR peaks of the NaCl aqueous solution trapped in the membrane shifted to lower field with increasing angle, \( \theta \), indicating the anisotropy of the silk fibroin membrane itself. The diamagnetic susceptibility of the membrane was calculated to be \(-0.64 \text{ ppm}\).

1. INTRODUCTION

Recently, it has been proved that Bombyx mori silk fibroin membrane is an excellent enzyme-immobilized membrane\(^1\)\textsuperscript{-3}. In technological application of the membranes to the use as biomaterials, it is necessary to obtain much information concerning the interaction between the silk fibroin and other molecules such as substrates, metal ions or water in the silk fibroin membrane\(^4\) as well as the structure of the membrane\(^5\)\textsuperscript{-10}).

In this paper, the physical state and orientation of the water trapped in porous silk fibroin membranes are studied in the absence and presence of metal ions by means of NMR spectroscopy. Especially, \( ^1H \) NMR spectral pattern of such water depends strongly on the angle between the membrane surface and the magnetic field, indicating the anisotropic state of the water molecules\(^1\textsuperscript{1}\),\textsuperscript{12}). In addition, the influence of alkali metal ions on the physical state of water\(^1\textsuperscript{3}) in the membrane is examined. These data are expected to be useful in development of the membranes for a bioreactor or biosensor because these membranes are usually used in aqueous solution and in some cases, in alkali metal aqueous solution.

2. EXPERIMENTAL

2.1 Materials

The porous B. mori silk fibroin membranes containing alkali metal ions were prepared as summarized in Fig. 1\textsuperscript{14}). The aqueous solution of the silk fibroin from B. mori cocoon was obtained as described elsewhere\(^1\textsuperscript{5}). The equivalent weight of polyethylene glycol, PEG with \( M_w=300 \) was added to the fibroin solution. After stirring carefully, the mixture was cast on acrylic resin plates at 20\( ^\circ \text{C} \) under 60\% relative humidity. The dried membrane containing PEG was soaked in distilled water for one day in order to remove PEG from the insolubilized membrane. Moreover, the porous membrane was soaked in a 3 M NaCl aqueous solution for one day to obtain the membrane containing the NaCl aqueous solution in the hole. After the membrane was taken out, the water attached to the membrane surface was...
wiped carefully with a filter paper. Similarly, the porous silk fibroin membranes containing 3 M LiCl or KCl aqueous solutions were obtained. The membranes prepared here were immediately used for NMR observation.

2.2 Measurements

$^1$H and $^{23}$Na NMR spectra of these membranes were observed by means of a JEOL FX-90Q spectrometer operating at 90 and 23.7 MHz, respectively at 25°C. To study the angular dependence of the spectra, the membranes were fixed by interposing with a Teflon frame. The membranes were aligned with its face oriented at different angles (10° interval) to the magnetic field as shown in Fig. 2). The $^1$H NMR spectra of water or $^{23}$Na NMR spectra of the NaCl aqueous solution trapped in the membrane were observed as a function of angles between the surface of the membrane and the magnetic field. The $^1$H NMR spin-lattice relaxation times of the water trapped in the membrane were determined at 25°C with usual inversion-recovery method.

3. RESULTS AND DISCUSSION

3.1 $^1$H NMR spectra of water in the porous silk fibroin membrane

$^1$H NMR spectra of the water trapped in the porous silk fibroin membrane in the absence of alkali metal ions were measured at $\theta = 90^\circ$, where $\theta$ is the angle between the surface of the membrane and the magnetic field (Fig. 3). The peaks observed were attributable to the water protons under the NMR experimental condition. At low content of water (8.6 w/w%), the spectra were broadened and apparently single. With increasing water content of the membrane, the doublets were observed. The $^1$H NMR spin-lattice relaxation time of each peak was determined for the membrane of water content, 83%. The values were 0.357 s and 0.362 s for each peak in the doublet.
Fig. 3. $^1$H NMR spectra of the porous $B. mori$ silk fibroin membrane containing 8.6% (a), 16% (b) and 93% (c) of water at the angle, $\theta = 90^\circ$, respectively. Dotted lines are simulated spectra assuming Lorentian on the basis of the observed spectrum (Fig. 4a) at $\theta = 50^\circ$, respectively, indicating same mobility of the individual peaks with each other.

Fig. 4 shows angular, $\theta$, dependences of the $^1$H NMR spectra of the water trapped in the porous silk fibroin membranes in the absence of alkali metal ions. At low content of the water (8.6%), the single peak shifted apparently to a lower field with changing $\theta = 0^\circ$ to $90^\circ$. On the other hand, in the case of the membrane of water content, 93%, the separation of the doublet depended on the angle $\theta$. This indicates that the direction of the water molecules trapped in the porous membrane is not completely averaged on the NMR time scale$^{11,12}$ although it is sure that fast exchange between free and bound waters on the NMR time scale occurs. These results suggest the presence of the strong interaction between silk fibroin and trapped water molecules. Strictly speaking, the shape of each peak of the doublet is slightly different from each other. The presence of a small peak at 0–0.1 kHz should be noted. The peak is angle $\theta$-independent and attributable to isotropic component of the water. Thus, this is one of the origins of departure from the symmetry of the doublet. In addition, the distortion of the membrane may also be such origins. Taking into account the appearance of the doublet for the membrane with water content 93%, the apparent single peak observed in Fig. 3a may be regarded as overlap of two peaks of the doublet; the each peak is broad and as a result the observed spectrum is apparently single. Actually, the peak width depends on the angle $\theta$, and becomes narrowest at $\theta = 50^\circ$ and we can reproduce the spectrum at $\theta = 90^\circ$ by overlapping of the spectrum at $\theta = 50^\circ$ as shown in Fig. 3a. When the water content of the membrane is 16%, the angular dependence of the spectrum was also observed. The appearance of the angle $\theta$-dependent doublet has been recently observed for the water molecules adsorbed in cellulose acetate membrane$^{12}$.

Figs. 5a, 5b and 5c also show the angular dependence of the $^1$H NMR spectra of the water molecules trapped in the porous fibroin membrane. Unlike the case of Fig. 4, samples contain alkali metal ions, NaCl, KCl and LiCl in the water. These spectra were doublet and shifted to the lower field with increasing angle $\theta$ from 0° to 90°. This trend was independent the species of metal ions and apparently the same as in Fig. 4b. The water contents were 74, 60 and 85% for the NaCl, KCl and LiCl membranes, respectively.
3.2 Analysis of the angular dependence of the $^1$H NMR peak separation

The separation of the doublet splitting, $\Delta(HH)$, due to the dipole-dipole interaction for a pair of protons was given by

$$\Delta(HH) = \frac{3}{4} (3\cos^2 \alpha - 1) L(HH) S_{22}$$

where, $\Delta(HH)$, $\alpha$ and $S_{22}$ mean the apparent separation width of the doublet peak (in Hz), the angle between the axis of proton-proton and the direction of the magnetic field and order parameter of proton-proton axis, respectively. The constant value, $L(HH)$ was reported to be approximately 60 kHz

The doublets observed in the $^1$H NMR spectra of water molecules trapped in the porous silk fibroin membranes are attributable to the dipole-dipole interaction of a pair of water protons as mentioned in the previous section. The values of the separation are determined from the peak simulation and are plotted against the angle, $\theta$ as shown in Fig. 6. In every cases, the change in the separation, $\Delta(HH)$ follows $(3\cos^2 \theta - 1)$ and $\Delta'(HH)$ becomes zero at $\theta = 50^\circ$. This trend is essentially independent of the presence and also the species of metal ions. These results indicate that the intramolecular proton-proton dipolar axis of the water molecules trapped in the porous silk fibroin membrane should be, on the average, parallel to the surface of the membrane. This is contrast to the angular dependent-$^1$H NMR of the water molecules absorbed in cellulose acetate film reported by Matsumura et al.$^{12}$). In their experi-
ment, the water contents were changed from 0.6% to 6.5% which were considerably lower than the contents in our experiments. In the case of such water molecules adsorbed in cellulose acetate, the proton-proton dipole axis tends to orient perpendicularly to the surface of the film because $\mathcal{A}(HH)$ becomes zero at $\theta = 30\degree - 40\degree$ and also the value of $\mathcal{A}(HH)$ at $\theta = 90\degree$ is approximately two times that at $\theta = 0\degree$. As shown in Fig. 4a, the doublet peaks were apparently no longer observed when the water content was 8.6% for silk fibroin membrane because of the peak broadening. This indicates that the interaction between water and silk fibroin membrane is considerably stronger than that in water-cellulose acetate film system.

When the observed angle $\theta$ equals the angle $\alpha$ in eq. (1), the value $\mathcal{A}(HH)$ at $\theta = 0\degree$ should be two times that at $\theta = 90\degree$. However, this prediction is in contrast to the observation that the values at $\theta = 0\degree$ and 90° are mostly same. At present, the reason of the discrepancy is not given clearly. However, the distribution of the proton-proton axis of water molecules with respect to the membrane surface is considered to be one of the origins. Thus, the order parameter, $S_{22}$, of the proton-proton axis of water molecules appeared in eq. (1) was obtained as 0.006 in the range of $\theta = 0\degree - 50\degree$, but 0.012 in the range of $\theta = 50\degree - 90\degree$. The former value, 0.006 is comparable to the $S_{22}$ value 0.0072 obtained for the water molecules adsorbed in cellulose acetate film although the water contents differ considerably with each other as mentioned above.

### 3.3 Diamagnetic susceptibility of the silk fibroin membrane

Fig. 7 shows $^{23}$Na NMR spectra of NaCl aqueous solution trapped in the porous silk fibroin membrane as a function of the angle, $\theta$. The single peak shifts gradually to the lower field with increasing angle from $0\degree$ to $90\degree$. This is clear from a comparison of the spectra of $\theta = 0\degree$ and $90\degree$. The amounts of the shift $\Delta \delta$ were plotted against $\theta$ in Fig. 8. Similar plots were obtained from Figs. 4 and 5 for the $^1$H NMR spectra of water trapped in the membrane and also included in Fig. 8. These plots coincide with each other with experimental error, indicating that such a shift is characteristic to the membrane itself rather than the species of the nuclei trapped in the membrane. Namely, the diamagnetic susceptibility $\chi_{\text{dia}}$ of the membrane is considered to be the origin and can be expressed by

$$
\Delta \delta(\theta) = 4\pi/3 (3 \cos^2 \theta - 1) \chi_{\text{dia}}. \tag{2}
$$
From Fig. 8, the $\chi_{\text{dia}}$ value was obtained $-0.64$ ppm. This absolute value was slightly larger than the value $0.53$ ppm reported for cellulose acetate membrane $^{12}$. Thus, these NMR observation is a simple method for determining the diamagnetic susceptibility of the membrane $^{17}$.

**REFERENCES**


**多孔質フィブロインのNMR 11. 多孔質フィブロイン膜中の水の配向に関する$^1$H NMRによる解析**

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多孔質フィブロイン膜中にトラップされた水の状態をNMR法により検討した。膜の含水量が高い場合、トラップされた水の$^1$H NMRビーグは、明瞭なダブレットとなり、その分離幅、ならびにビーグ位置は、膜表面と膜中にとまる角度、$\theta$に関係依存した。この傾向は、その水中アルカリ金属イオンが存在するか否かに依存しない。その分離幅と角度、$\theta$間の詳細な検討から、トラップされた水の$^1$H-H軸の配向は、分布しているものの、平均としてその配向方向は、膜平面に平行になっていると考えられた。膜中にトラップされた水の$^1$H NMRビーグ、ならびにNaCl水溶液の$^{23}$Na NMRビーグは、角度、$\theta$の増加について、低磁場シフトした。これは、膜自身の異方性に起因し、そのシフト量から、膜の反磁性磁化率を$-0.64$ ppmと見積った。