Crystal transition between hydrate and anhydrous β-chitin monitored by synchrotron X-ray fiber diffraction

Kayoko Kobayashi [a], Satoshi Kimura [a], Eiji Togawa [b], Masahisa Wada [a,*]

[a] *Department of Biomaterials Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan*
[b] *Forestry and Forest Products Research Institute, Matsunosato 1, Tsukuba, Ibaraki 305-8687, Japan*

ARTICLE INFO

ABSTRACT

The oriented fibers of highly crystalline β-chitin were prepared from tubes of *Lamellibrachia satsuma* that had never been dried. Synchrotron radiation X-ray fiber diffraction of these never-dried samples revealed that the native β-chitin could be fully described by a one-chain monoclinic unit cell, a = 4.80 Å, b = 11.15 Å, c (fiber repeat) = 10.44 Å, and γ = 96.39°, with the space group being $P2_1$. On the basis of the unit-cell volume, the asymmetric unit is composed of one *N*-acetyl-ᴅ-glucosamine residue and two water molecules, indicating that this native β-chitin is the dihydrate form. The crystal transition between the dihydrate and anhydrous forms was also monitored by synchrotron X-ray diffraction under controlled relative humidity (R.H.). The transition from the dihydrate to the anhydrous form occurred at R.H. 30–20% in the drying process. On the other hand, the anhydrous form converted into the monohydrate form around R.H. 70% in the wetting process, but did not return to the initial dihydrate form even at R.H. 100%. By dropping water, however, the monohydrate form was converted into the dihydrate form. Thus the crystal transition between the dihydrate and anhydrous forms was shown to take place reversibly through an intermediate structure, the monohydrate form, showing a large hysteresis.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Chitin, composed of β-1,4-linked *N*-acetyl-ᴅ-glucosamine residues, is one of the most abundant biopolymers on earth, occurring in nature as crystalline fibrils (Blackwell, 1973; Rudall, 1963). The chemical structure of chitin is the same as that of another abundant natural polymer, cellulose, except for an acetamido group in chitin and a hydroxyl group in cellulose on the ring C2 position. In addition, they have similar conformations of the twofold ribbon-like helix. The naturally occurring polymorphs of chitin are designated α- and β-chitin, which are readily distinguished on the basis of their different X-ray diffraction patterns and infrared spectra (Blackwell, 1973; Rudall, 1963).

α-Chitin is a thermodynamically stable and abundant form of chitin. It occurs in the cuticles of crustaceans and insects, in cell walls of fungi, and in other organisms. X-ray diffraction studies have revealed that α-chitin adopts a two-chain orthorhombic unit cell with the space group $P2_12_12_1$, indicating an antiparallel chain arrangement (Minke & Blackwell, 1978; Sikorski, Hori, & Wada, 2009). This model was further supported by an electron diffraction study using highly crystalline samples from the grasping spines of the arrow worm *Sagitta* sp (Saito, Okano, Chanzy, & Sugiyama, 1995).

On the other hand, β-chitin is a rather rare form occurring in squid pens, spines of some centric diatoms, and tubes of pogonophores and vestimentiferans, and in some other organisms (Blackwell, 1969; Rudall, 1963). In the structures of β-chitin, it was reported that an anhydrous form and more than two hydrate forms exist (Blackwell, 1969; Gaill, Persson, Sugiyama, Vuong, & Chanzy, 1992; Rössle et al., 2003; Saito, Kumagai, Wada, & Kuga, 2002; Tanner, Chanzy, Vincendon, Roux, & Gaill, 1990). Among them, only the crystal structure of β-chitin anhydrous has been determined by X-ray diffraction studies. The unit cell of β-chitin anhydrous is a one-chain monoclinic structure, a = 4.85 Å, b = 9.26 Å, c (fiber repeat) = 10.38 Å, and γ = 97.5°, with the space group being $P2_1$ (Gardner & Blackwell, 1975). The chitin chains in this anhydrous form therefore are packed in a parallel arrangement, as opposed to the antiparallel arrangement in α-chitin. A structural study proposed that β-chitin anhydrous adopts a molecular sheet in the *ac*-plane that is formed by hydrophobic forces of glucopyranoside rings and by intermolecular hydrogen bonds C=O···H–N and C=O···H–O6. These sheets are stacked because of hydrophobic forces, and thus no hydrogen bond exists between the sheets along the [0 1 0] direction (Fig. 1) (Blackwell, 1969; Gardner & Blackwell, 1975; Saito et al., 2002).

As opposed to the quite stable α-chitin, β-chitin possesses unique properties that it incorporates small molecules into the crystalline lattice to form various crystalline complexes, crystallo-
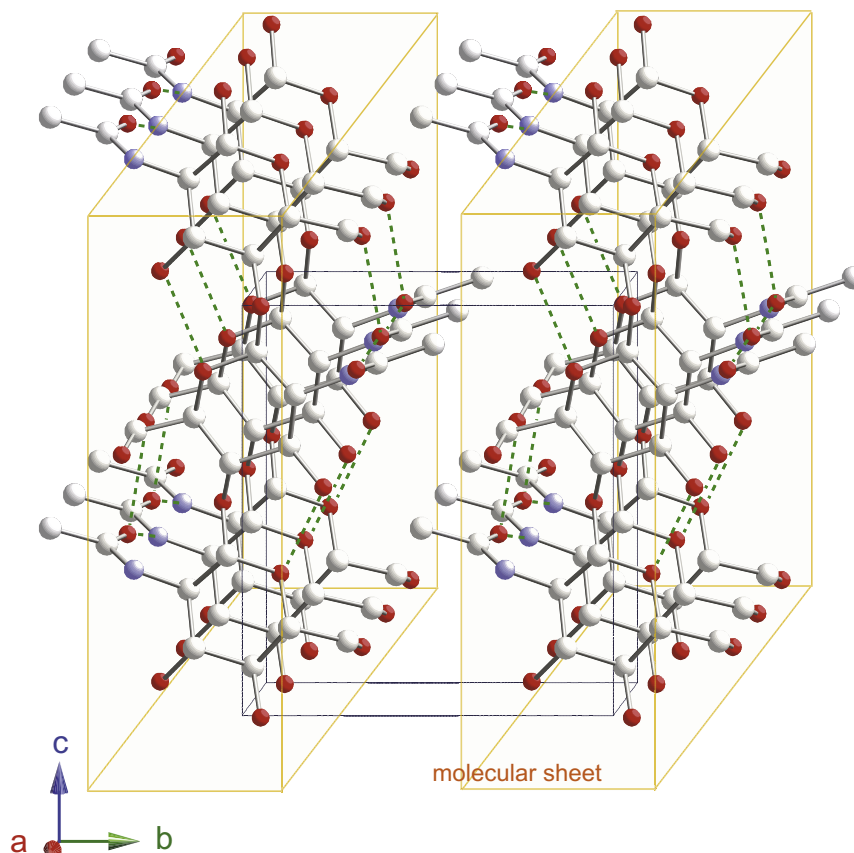
---

* Corresponding author. Tel.: +81 3 5841 5247; fax: +81 3 5841 2677.
E-mail address: awadam@mail.ecc.u-tokyo.ac.jp (M. Wada).

Fig. 1. Crystal structure of anhydrous β-chitin viewed perpendicular to the *c*-axis direction with the *c*-axis slightly tilted to show the intermolecular hydrogen bonds. The chitin molecular sheet almost parallel to the *ac*-plane is formed by hydrophobic forces between glucosamine rings and further stabilized by intermolecular bonds. No intermolecular hydrogen bonds exist between the molecular sheets. Broken lines indicate hydrogen bonds.

solvates (Noishiki, Kuga, Wada, Hori, & Nishiyama, 2004; Noishiki, Nishiyama, Wada, Okada, & Kuga, 2003; Saito, Okano, Gaill, Chanzy, & Putaux, 2000; Saito, Okano, Putaux, Gaill, & Chanzy, 1998). The most important and readily formed complexes are the hydrates. When β-chitin swells in water, the molecular sheets (Fig. 1) remain intact but move apart to intercalate water molecules. The early X-ray diffraction studies identified anhydrous, monohydrate, and dihydrate forms (Blackwell, 1969). The *d*-spacings of the innermost equatorial reflections corresponding to the distances between the molecular sheets are in the order dihydrate > monohydrate > anhydrous forms. However, mixed patterns of the hydrate forms were sometimes recorded, probably because of the use of drying samples before wetting and/or experimental conditions. Native β-chitin should be a hydrate form, because it is synthesized by living organisms under water. As of today, however, neither the unit-cell parameters nor the structure of any of these hydrates has been described with certainty. Therefore, we collected highly crystalline β-chitin samples of tubeworm *Lamellibrachia*; the samples kept in moist conditions were investigated using synchrotron X-ray fiber diffraction and solid-state cross-polarization/magic angle spinning (CP/MAS) ^{13}C NMR spectroscopy to reveal the hydrate form of β-chitin.

The transitions between the hydrate and anhydrous forms take place rather easily through drying and wetting (Rössle et al., 2003; Saito et al., 2002). However, the nature of the transitions has not been fully understood. In this study, we further monitored the crystal transition between the native hydrate and anhydrous forms under controlled relative humidity (R.H.) using synchrotron X-ray diffraction.

2. Experimental

2.1. Samples

Satsuma tubeworms (*Lamellibrachia satsuma*) were collected from the sea off Kagoshima Bay at a depth of about 100 m using a remotely operated vehicle, Hyper-Dolphin (JAMSTEC, Japan). The bodies of the tubeworms were removed by washing the tube with water, and the tubes were kept in deionized water in a refrigerator. The never-dried tubes kept in deionized water were cut into approximately 3 cm lengths and were then deproteinized according to the method described previously. After overnight immersion in 1 N NaOH solutions at room temperature, they were treated with 0.3% NaClO₂ solutions buffered at pH 4.9 in 0.1 M acetate buffer at 70 °C for 3 h (Sugiyama, Persson, & Chanzy, 1991). The purified samples were stored in deionized water containing 0.01% NaN₃ in a refrigerator until use.

2.2. Synchrotron X-ray fiber diffraction

Oriented thin lamellae were peeled off from the purified tube by tweezers in deionized water. They were pulled by hanging small weights at one end in a water-saturated atmosphere in a desiccator to achieve high orientation of chitin microfibrils along the pulling direction. The oriented fibers about 200 μm in thickness were clamped on holders in water. The native hydrate samples were maintained in water until just before the X-ray measurement. The clamped samples were dried over P₂O₅ in a desiccator to prepare the anhydrous form, and the dried samples were subse-

quently stored over saturated solutions of K_2SO_4 (R.H. 98%) in a desiccator to prepare the rehydrated form.

Synchrotron X-ray fiber diffraction was carried out at the beam line BL38B1 at SPring-8 (Hyogo, Japan). Three oriented fibers thus prepared were each mounted on a goniometer head and synchrotron radiated X-rays ($\lambda = 1.0$ Å) were orthogonally irradiated for 120 s to the fiber axis under 1 L/min of humidity-controlled air flow generated by a humidity generator (Shinyei SRG-1R): R.H. 100% for native hydrate and rehydrated forms, and R.H. 0% for the anhydrous form. The fiber patterns were recorded using a camera system equipped with a flat imaging plate (IP) (R-Axis V, Rigaku). The sample-to-IP distance was calibrated using Si powder ($d = 0.31355$ nm) (Sikorski et al., 2009; Wada, Kwon, & Nishiyama 2008). Peak positions up to fourth layer lines were measured using R-Axis display software (Rigaku). After indexing the d-spacings, unit-cell parameters were determined by a least-squares method (Sikorski et al., 2009).

2.3. Solid-state CP/MAS ^{13}C NMR spectroscopy

The purified samples were homogenized into small fragments using a double-cylinder-type homogenizer. The suspensions were centrifuged at 5000g for 5 min, and the precipitate was kept in a wet condition until the NMR measurement for the native hydrate form. To prepare the anhydrous form, the precipitate was freeze-dried and further dried over P_2O_5 in a desiccator. The rehydrated form was prepared from anhydrous samples by keeping for more than one week in a desiccator with saturated K_2SO_4 solutions.

Solid-state ^{13}C NMR spectra of three types of samples, native hydrate, anhydrous, and rehydrated forms, were obtained on a CMX 300 spectrometer (Chemagnetics), operating at a ^{13}C NMR frequency of 75.6 MHz. The samples in a 7.5 mm zirconia rotor for the CMX300 were spun at 3.5–4.5 kHz in a solid-state probe at the magic angle. All spectra were obtained at room temperature using ^{1}H NMR 90° pulse lengths of 4.8 or 5.0 ms, with a cross-polarization time of 1.0 ms and 60 kHz CW proton decoupling. A recycle time of 3 s was used. The spectra were calibrated using adamantane as a standard (the CH_2 peak at 29.5 ppm gives shift values referenced to the TMS carbon at 0 ppm). The rotor was sealed by a Teflon cap to avoid drying of the sample during the measurement when measuring the samples of hydrate and rehydrated forms.

2.4. Monitoring of the crystal transition by synchrotron X-ray diffraction

Monitoring of the crystal transition between hydrate and anhydrous forms by X-ray diffraction was also performed at the beam line BL38B1 at SPring-8 (Hyogo, Japan) with the same experimental setup as the above fiber diffraction section. Oriented fibers prepared from never-dried samples were also used. The fiber diffraction diagrams at each R.H. from 90% to 0% with a stepwise decrease of 10% were recorded, where the X-ray exposure time was 2 min and the interval between each step was 15 min. After recording at R.H. 0%, the fiber diffraction diagrams with a 10% stepwise increase of R.H. were subsequently carried out up to 100% using the same sample as for the drying process. Finally, about 100 μL of water were dropped onto the specimen and then the fiber diffraction diagram was recorded. The temperature was not controlled but the monitored temperature was about 23 °C during the X-ray diffraction experiments.

Equatorial and meridional X-ray diffraction profiles at each R.H. were obtained by analyzing the fiber diffraction diagrams using R-Axis display software (Rigaku). The peak positions were determined by peak-fitting of the X-ray diffraction profiles as previously reported (Wada, Okano, & Sugiyama, 1997; Wada et al., 2008). The

unit-cell parameters were refined by the least-squares method from the d-spacings and their indices.

2.5. Water desorption and adsorption measurements

Water desorption/adsorption isotherms of β-chitin were determined by keeping samples over saturated salt solutions in desiccators at 23 °C. The never-dried sample was used for desorption measurements and the sample dried over P_2O_5 was used for adsorption measurements.

3. Results and discussion

3.1. Synchrotron X-ray fiber diffraction

Three crystal forms of β-chitin could be prepared from never-dried samples by drying and subsequent wetting treatments. Fig. 2a shows the X-ray fiber diffraction diagram of the native hydrate form recorded from the never-dried samples at R.H. 100%. The diagram shows high resolution; each reflection is very sharp but with some azimuthal distribution. All of the reflections in the diagram could be indexed according to a one-chain monoclinic unit cell with dimensions $a = 4.80$ Å, $b = 11.15$ Å, c (fiber repeat) = 10.44 Å, and γ (monoclinic angle) = 96.39°. On the meridian of the diagram 0 0 l reflections where l is odd were absent, indicating that the space group was $P2_1$ with the 2_1-axis along the fiber axis. The pair of strong 0 0 2 and weak 0 0 4 reflections shows a characteristic feature that the β-chitin hydrate form does not have staggering along the fiber axis, the same as the proposed structure of β-chitin anhydrous (Gardner & Blackwell, 1975). This indicates that one N-acetyl-D-glucosamine residue is contained in the asymmetric unit. The number of water molecules included in the asymmetric unit will be discussed below.

The native hydrate form could be readily converted into the anhydrous form by drying. An X-ray fiber diffraction diagram of the anhydrous form is shown in Fig. 2b. The diagram shows some different features in each layer line from that of the native hydrate form. The unit cell of the anhydrous form could be determined from the diagram: one-chain monoclinic with dimensions $a = 4.82$ Å, $b = 9.20$ Å, $c = 10.38$ Å, and $\gamma = 97.05°$. On the meridian of the diagram, the 0 0 1 reflection is weak, 0 0 2 is very strong, 0 0 3 is very weak, and 0 0 4 is medium. The intensity distribution of these reflections indicates that the space group is $P2_1$. A one-chain monoclinic unit cell with $P2_1$ symmetry suggests that only one N-acetyl-D-glucosamine residue is in the asymmetric unit. This agrees with the proposed structure of anhydrous β-chitin (Gardner & Blackwell, 1975).

Unit-cell parameters of the native hydrate and anhydrous forms gave insight into the intercalating behavior of the water molecules. Although the lengths of the a- and c-axes were almost the same, within a 1% difference, the b-axis was significantly different in the two forms; the length of the native hydrate form was about 20% longer than that of the anhydrous form. This anisotropic change of unit-cell parameters by drying suggests that the molecular sheet in the ac-plane would also be retained in the native hydrate form, but separated by the intercalating water molecules along the b-axis (Fig. 1). From the unit-cell parameters, the unit-cell volumes of native hydrate and anhydrous forms were 555 and 457 Å^3, respectively. From the difference of these unit-cell volumes and the density of water, 1 g/cm^3, the number of water molecules included in the unit cell of the native hydrate form was calculated to be 3.4. Taking into account the monoclinic $P2_1$ symmetry, we estimated that four water molecules were incorporated into the one-chain unit cell. The difference of 0.6 would be ascribed to the denser packing of water molecules in the native hydrate
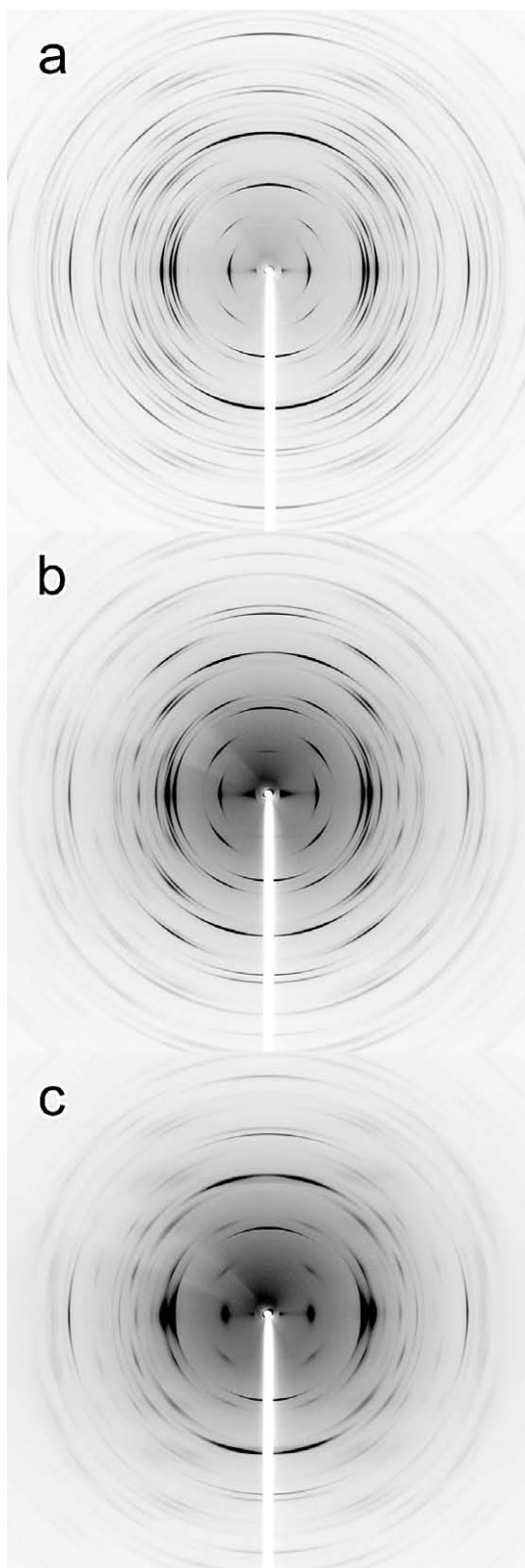
Fig. 2. Synchrotron X-ray fiber diffraction diagrams of β-chitin native hydrate (a), anhydrous (b), and rehydrated forms (c). The fiber axis is vertical. The native hydrate and rehydrated were shown to be the dihydrate and monohydrate forms, respectively.

Nishiyama, & Langan, 2006; Wada et al., 2008). Therefore, the asymmetric unit of the native hydrate form would contain one N-acetyl-D-glucosamine residue and two water molecules, that is, intact β-chitin is the dihydrate form.

The anhydrous form was converted into the rehydrated form by conditioning at an atmosphere of R.H. 100%. An X-ray fiber diffraction diagram of the rehydrated form is shown in Fig. 2c. The diagram is quite similar to that of the native hydrate form (Fig. 2a), but the reflections have became somewhat broad and those in the wide-angle region were somewhat reduced, indicating a slight disruption of the highly ordered state by the deswelling and swelling processes. The innermost reflection on the equator appeared at $d = 10.28$ Å, which is between 0 1 0 of the native hydrate and anhydrous forms. This means that the rehydrated form is another hydrate form containing fewer water molecules than the native hydrate form. On the meridian of the diagram, the odd 0 0 l reflections were absent, 0 0 2 is very strong, and 0 0 4 is weak. This indicates that the rehydrated form is a structure that shows no stagger along the chitin fiber axis with $P2_1$ symmetry. Thus, we tried to determine the unit-cell parameters according to the one-chain monoclinic system, but this attempt failed.
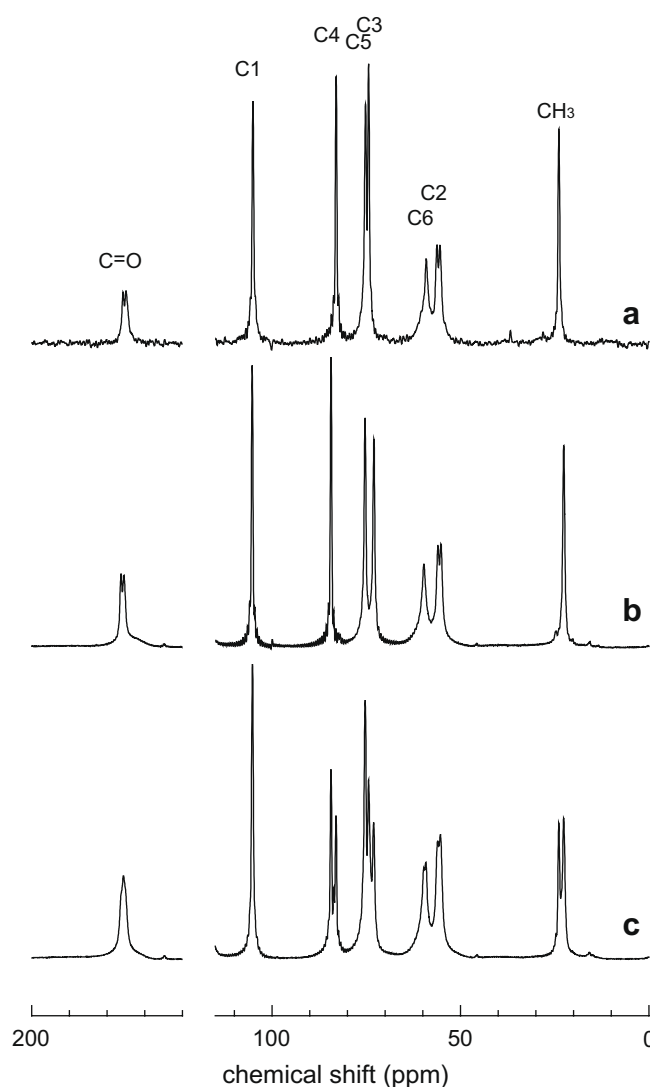

Fig. 3. Solid-state CP/MAS ^{13}C NMR spectra of β-chitin; samples (a–c) are the same as Fig. 2. The assignment of the carbon atoms is derived from Tanner et al. (1990).

form. Such a small difference between the estimated free volume and X-ray structure was also reported in the number of ammonia molecules included in the cellulose I–ammonia complex (Wada,

Table 1
^{13}C chemical shifts recorded from native hydrate, anhydrous, and rehydrated forms of β-chitin.

Sample	Chemical shifts (ppm)							
	C1	C2	C3	C4	C5	C6	CH$_3$	C=O
Native hydrate	105.1	55.4	74.4	83.0	75.3	59.1	23.9	174.9
		56.3						175.8
Anhydrous	105.3	55.2	73.0	84.4	75.4	59.8	22.6	175.5
		56.0						176.4
Rehydrate	105.2	55.3	73.1	83.1	75.3	59.1	22.6	175.5
		56.2	74.3	84.4		59.6	23.9	176.4

3.2. Solid-state ^{13}C NMR spectroscopy

Solid-state ^{13}C NMR spectra of the native hydrate, anhydrous, and rehydrated forms of β-chitin corresponding to the samples used for synchrotron X-ray fiber diffraction are shown in Fig. 3. Chemical shifts of each carbon atom were determined from the spectra and are listed in Table 1. The common features of the spectra of native hydrate and anhydrous forms (Fig. 3a and b) are that line widths are remarkably small and each carbon atom appeared as a singlet except for C2 and the carbonyl carbon, which were asymmetric doublets. These splittings of C2 and the carbonyl carbon peaks would arise from ^{13}C–^{14}N interactions for the ^{13}C atoms that are directly bound to the ^{14}N atom of the acet-amido group (Tanner et al., 1990). Thus, each carbon atom should be equivalent, which indicates that the native hydrate and anhydrous forms are composed of only one symmetrically independent *N*-acetyl-ᴅ-glucosamine residue. This is consistent with the above X-ray fiber diffraction analysis. The chemical shifts of native

hydrate and anhydrous forms for each carbon atom are very close to each other (Table 1); the differences are less than 1.4 ppm. This result implies that both forms have a similar conformation of the molecules. Upon drying, the native hydrate form would release water molecules from between the chitin molecular sheets with-out changing the sheet structures. In the spectrum of the rehy-drated form, however, the peaks corresponding to C3, C4, C6, and methyl carbon also appeared as doublets (Fig. 3c). In addition, all of the chemical shifts were almost in the middle or almost the same as those of the native hydrate and anhydrous forms (Table 1). This implies that two independent *N*-acetyl-ᴅ-glucosa-mine residues should be contained in the asymmetric unit. Com-bined with the result of the X-ray fiber diffraction, the rehydrated form should be an intermediate structure between the native hy-drate and anhydrous forms with a larger unit cell, probably con-taining two chains, and the conformation of one-chain similar to that of the native hydrate form and the other similar to the anhy-drous form.
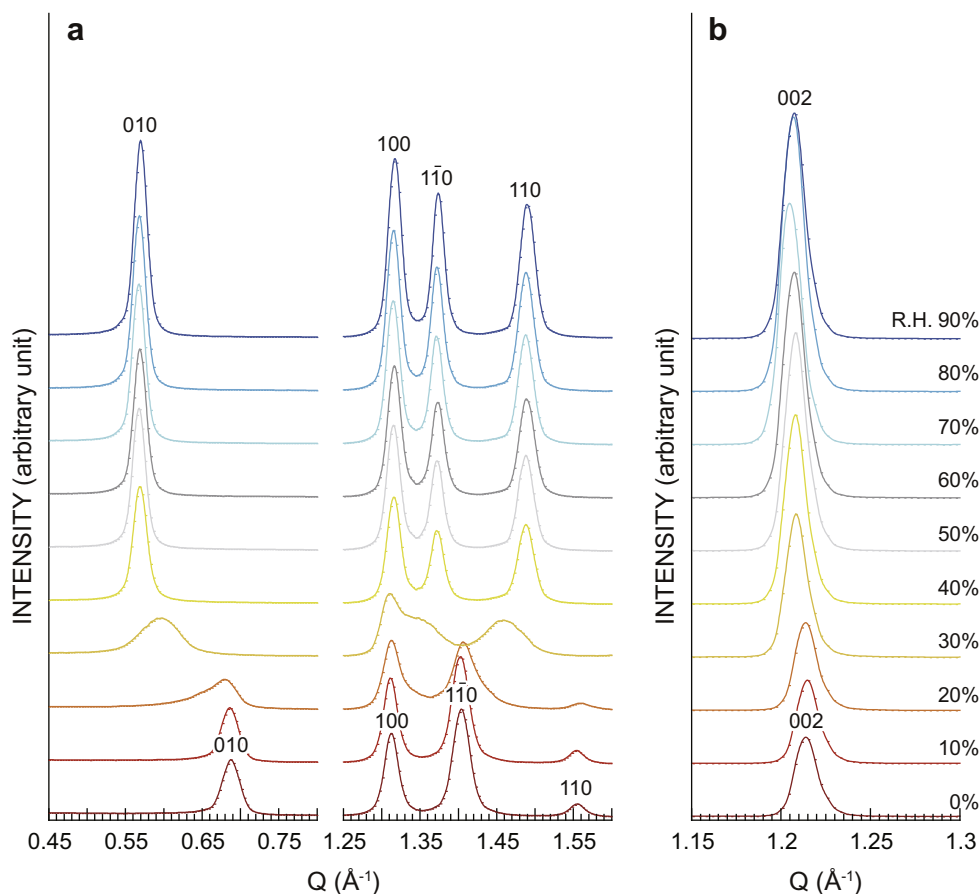

Fig. 4. Changes in equatorial (a) and meridional (b) X-ray diffraction profiles of the native hydrate form of β-chitin during the drying process. Q is the scattering vector (2π/d).

The C6 chemical shifts of the β-chitin samples are in the range of 59.1–59.8 ppm (Table 1). Horii, Hirai, and Kitamaru (1983) suggested that a correlation exists between the C6 chemical shifts and the conformation of the primary hydroxyl group. This correlation indicates that the conformations of hydroxymethyl groups of the native hydrate, anhydrous, and rehydrated forms adopt the gg conformation (Sundaralingam 1986). In the case of the anhydrous form, this is in agreement with the proposed X-ray structure (Gardner & Blackwell, 1975).

3.3. Crystal transition between hydrate and anhydrous forms

The crystal transition from the native hydrate form to the anhydrous form was monitored by synchrotron X-ray diffraction under controlled R.H. at 23 °C. Fig. 4 shows equatorial and meridional X-ray diffraction profiles of the drying process from R.H. 90% to 0%. Four equatorial peaks, indexed as 0 1 0, 1 0 0, 1 $\bar{1}$ 0, and 1 1 0, and one meridional peak indexed as 0 0 2 were observed in the Q ($= 2\pi/d$) ranges of 0.45–1.6 and 1.15–1.3 Å^{-1}, respectively. Calculated d-spacings were $d_{010} = 11.03$ Å, $d_{100} = 4.77$ Å, $d_{1\bar{1}0} = 4.57$Å, $d_{110} = 4.22$ Å, and $d_{002} = 5.20$ Å, which agree with the d-spacings calculated from the unit cell of the native hydrate form. Although all of the peaks gradually got broader with decreasing R.H., their positions did not change until R.H. 40%. When the R.H. reached 30%, the 0 1 0 peak shifted to the high Q range and the 1 $\bar{1}$ 0 and 1 1 0 peaks shifted to the low Q range with considerable broadening, but the 1 0 0 and 0 0 2 peaks still stayed in almost the same position. The d-spacing of 0 1 0, $d_{010} = 10.56$ Å, was almost the same as that of the rehydrated form observed by fiber diffraction

(Fig. 2c), suggesting that the intermediate structure between the native hydrate and anhydrous forms is a hydrate form corresponding to the rehydrated form. At R.H. 20%, four equatorial peaks that correspond to the anhydrous form appeared and the transition to the anhydrous form was almost complete. By drying at R.H. 0%, the profile became typical of the anhydrous form, and each peak became sharper than that at R.H. 20%. The d-spacings of the five equatorial and meridional peaks were $d_{010} = 9.14$ Å, $d_{100} = 4.79$ Å, $d_{1\bar{1}0} = 4.48$Å, $d_{110} = 4.04$ Å, and $d_{002} = 5.18$ Å. These values agree with the d-spacings calculated from the unit cell of the anhydrous form. In the drying process of the never-dried β-chitin samples, the native hydrate form was stable to R.H. 40%, the intermediate structure of the rehydrated form was observed at R.H. 30%, and the transition to the anhydrous form was achieved at R.H. 20%.

After drying to R.H. 0%, the crystal transition of β-chitin from anhydrous to hydrate forms was successively monitored by synchrotron X-ray diffraction. Fig. 5 shows equatorial and meridional X-ray diffraction profiles of the wetting process from R.H. 0% to 100%. Although the peaks broadened, their positions did not change from R.H. 0% up to 70%, indicating that the anhydrous form is stable in this humidity range in the wetting process. At R.H. 80%, the peaks became much broader, and the 010 peak slightly shifted to the low Q range. The peak broadening with increasing R.H. indicates that the anhydrous form became disordered with a smaller crystallite size because of the irregularly intercalated water molecules in the crystallites. Above R.H. 80%, the 010 peak further shifted with increasing R.H., and its d-spacing was $d_{010} = 10.28$ Å at R.H. 100%. The equatorial profile at R.H. 100% was the same as the profile of R.H. 30% in the drying process (Fig. 4), indicating that
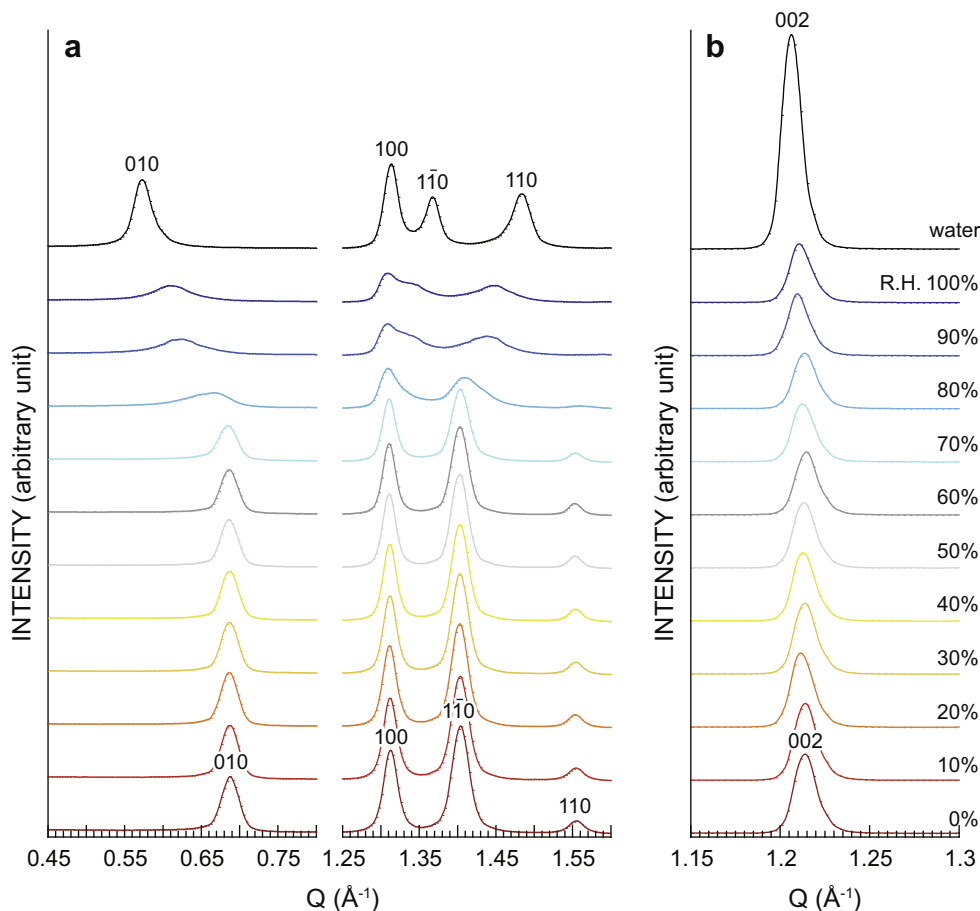

Fig. 5. Changes in equatorial (a) and meridional (b) X-ray diffraction profiles of the anhydrous form of β-chitin during the wetting process. Q is the scattering vector ($2\pi/d$).

the anhydrous form could not be converted into the native hydrate form even in an atmosphere of R.H. 100%. The structure was the same as the rehydrated form of the X-ray fiber diffraction diagram (Fig. 2c).

After the measurement at R.H. 100%, a few drops of water were put on the sample and then X-ray diffraction of the sample was carried out. The equatorial and meridional profiles are shown at the top of Fig. 5. The profiles were the same as those of the initial hydrate form (Fig. 4 top); however, those peaks were broad, showing that the rehydrated form was immediately converted into the native hydrate form by dropping water. The peak broadening indicates that chitin microcrystals were somewhat broken or damaged during the drying and wetting processes. In the wetting process of the dried β-chitin samples, the anhydrous form was stable up to R.H. 70–80%, the rehydrated form was observed at R.H. 90–100%, and conversion into the native hydrate form was accomplished by dropping water on the sample. Taking together the results of drying and wetting processes, the transition between the native hydrate and the anhydrous forms reversibly occurred by water adsorption and desorption through the intermediate structure, rehydrated form, showing a large hysteresis.

The unit-cell parameters, the *a*-, *b*-, and *c*-axes, and monoclinic angle γ, were calculated from the profiles shown in Figs. 4 and 5 according to the one-chain monoclinic model. These unit-cell parameters and the volume of the unit cell at each R.H. are shown in Fig. 6. The *a*-axis was almost constant during the measurement. On the other hand, the *b*-axis showed a large change at transition points: decreasing and increasing in the drying and wetting processes, respectively. The *c*-axis similarly changed at the transition points, but the magnitude of the change was much smaller than that of the *b*-axis. The monoclinic angle also changed, but the difference was less than 1°. These results clearly demonstrated that transition between native hydrate and anhydrous forms was not only a change in the *b*-axis but also in other unit-cell parameters. Intercalated water molecules would affect the structure of molecular sheets in the *ac*-plane. The transition between hydrate and anhydrous forms may not fully explain the separating and approaching of the molecular sheets by intercalating water molecules as previously reported by Saito et al. (2002). Further experiments are required to elucidate the transition mechanism.

To estimate the number of water molecules in the native hydrate and rehydrated forms, changes in weight of samples during drying and wetting processes were also measured. The desorption/adsorption isotherm is shown in Fig. 7. Combined with the results of X-ray diffraction measurements, the decrease in weight from the beginning of drying to R.H. 60–50% was due to the desorption of water from the crystallite surface. On the other hand, the subsequent decrease from R.H. 50% to 20% was mainly due to a release of water molecules from the crystalline lattice accompanied by the transition from the native hydrate to the anhydrous form. The loss of weight was approximately 18% of the anhydrous form dried at R.H. 0%, which corresponds to two water molecules per *N*-acetyl-D-glucosamine residue. This result is consistent with the value calculated from the difference in unit-cell volumes.

In the water adsorption process, the weight increased by approximately 12% above R.H. 70–80%. This gain in weight corresponds to 1.5 water molecules per *N*-acetyl-D-glucosamine residue, however, that quantity can include additional water molecules bound to the surface of individual crystallites but not part of the crystalline lattice. Therefore, we assumed that one water molecule per *N*-acetyl-D-glucosamine residue was incorporated in the rehydrated form.

In our experiments, the monohydrate form could be observed as an intermediate phase during the transition between the dihydrate and anhydrous forms in both drying and wetting processes. There have been some attempts to determine the nature of the transition,
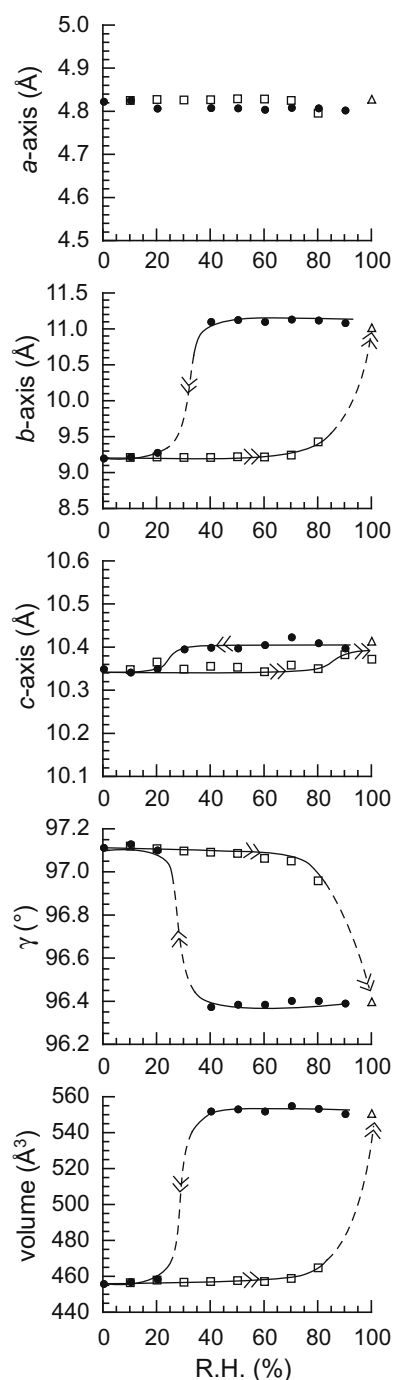

Fig. 6. Changes in unit-cell parameters and volumes of β-chitin in the drying process (filled circles) and wetting process (open squares), calculated using the peaks shown in Figs. 4 and 5. The open triangles show the values that were obtained by dropping water onto the sample at the end of the wetting process. The dashed lines show the region of transitions.

but the monohydrate form has not been observed alone in those studies (Rössle et al. 2003; Saito et al. 2002). Rössle et al. (2003) monitored the hydration induced by dropping water onto the dried samples. However, the transition from the anhydrous to the dihydrate form occurred so fast with enough water that the monohydrate form did not appear in the hydration process. Saito et al. (2002) studied the thermally induced transition in water by DSC and X-ray diffraction. In spite of the two clear endothermic peaks on heating in the DSC thermogram, only mixed patterns of hydrate and anhydrous forms were obtained in the X-ray measurement,
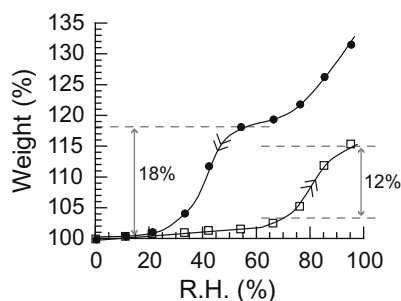
Fig. 7. Water desorption and adsorption isotherms of β-chitin starting from the never-dried sample. The filled circles and open squares show the values in the drying process and wetting process, respectively, as in Fig. 6.

probably because of the experimental conditions. On the other hand, we monitored the equilibrium state at each R.H. Under these experimental conditions, water molecules should be released/intercalated more gradually than the transitions induced by dropping water or heating in water (Rössle et al. 2003; Saito et al. 2002). This would be a reason why we could observe the intermediate phase, the monohydrate form.

In addition, X-ray diffraction under controlled R.H. has the advantage that the number of intercalating water molecules could be estimated by combining with isotherm measurements, as in the present study. There are many polysaccharides known to exist as hydrate forms such as cellulose, amylose, $(1 \rightarrow 3)$-β-D-glucan, $(1 \rightarrow 3)$-β-D-xylan, $(1 \rightarrow 4)$-β-D-xylan, nigeran, mannan, etc. However, their transition mechanisms and the roles of water molecules in the crystalline hydrate structures have not been fully understood. Further experiments applying the methods of this study can contribute to elucidating the nature of hydration of polysaccharides.

Acknowledgments

References

Blackwell, J. (1969). Structure of β-chitin or parallel chain systems of poly-b-$(1 \rightarrow 4)$-N-acetyl-D-glucosamine. *Biopolymers, 7,* 281–298.
Blackwell, J. (1973). The polysaccharides. In A. G. Walton & J. Blackwell (Eds.), *Biopolymers* (pp. 464–513). New York & London: Academic Press.
Gaill, F., Persson, J., Sugiyama, J., Vuong, R., & Chanzy, H. (1992). The chitin system in the tubes of deep sea hydrothermal vent worms. *Journal of Structural Biology, 109,* 116–128.
Gardner, K. H., & Blackwell, J. (1975). Refinement of the structure of β-chitin. *Biopolymers, 14,* 1581–1595.
Horii, F., Hirai, A., & Kitamaru, R. (1983). Solid-state ^{13}C-NMR study of conformations of oligosaccharides and cellulose – conformation of CH_2OH group about the exo-cyclic C–C bond. *Polymer Bulletin, 10,* 357–361.
Minke, R., & Blackwell, J. (1978). The structure of α-chitin. *Journal of Molecular Biology, 120,* 167–181.
Noishiki, Y., Kuga, S., Wada, M., Hori, K., & Nishiyama, Y. (2004). Guest selectivity in complexation of β-chitin. *Macromolecules, 37,* 6839–6842.
Noishiki, Y., Nishiyama, Y., Wada, M., Okada, S., & Kuga, S. (2003). Inclusion complex of β-chitin and aliphatic amines. *Biomacromolecules, 4,* 944–949.
Rössle, M., Flot, D., Engel, J., Burghammer, M., Riekel, C., & Chanzy, H. (2003). Fast intracrystalline hydration of β-chitin revealed by combined microdrop generation and on-line synchrotron radiation microdiffraction. *Biomacromolecules, 4,* 981–986.
Rudall, K. M. (1963). The chitin/protein complexes of insect cuticles. *Advanced in Insect Physiology, 1,* 257–313.
Saito, Y., Kumagai, H., Wada, M., & Kuga, S. (2002). Thermally reversible hydration of β-chitin. *Biomacromolecules, 3,* 407–410.
Saito, Y., Okano, T., Chanzy, H., & Sugiyama, J. (1995). Structural study of α chitin from the grasping spines of the arrow worm (*Sagitta* spp.). *Journal of Structural Biology, 114,* 218–228.
Saito, Y., Okano, T., Gaill, F., Chanzy, H., & Putaux, J.-L. (2000). Structural data on the intra-crystalline swelling of β-chitin. *International Journal of Biological Macromolecules, 28,* 81–88.
Saito, Y., Okano, T., Putaux, J.-L., Gaill, F., & Chanzy, H. (1998). Crystallosolvate of β chitin and alcohols. In A. Domard, G. A. F. Roberts, & K. M. Vårum (Eds.), *Advances in chitin science* (vol. 2, pp. 507–512). Lyon, France: Jacques André Publisher.
Sikorski, P., Hori, R., & Wada, M. (2009). Revisit of α-chitin crystal structure using high resolution X-ray diffraction data. *Biomacromolecules, 10,* 1100–1105.
Sugiyama, J., Persson, J., & Chanzy, H. (1991). Combined infrared and electron-diffraction study of the polymorphism of native celluloses. *Macromolecules, 24,* 2461–2466.
Sundaralingam, M. (1986). Some aspects of stereochemistry and hydrogen bonding of carbohydrates related to polysaccharide conformations. *Biopolymers, 6,* 189–213.
Tanner, S. F., Chanzy, H., Vincendon, M., Roux, J. C., & Gaill, F. (1990). High-resolution solid-state carbon-13 nuclear magnetic resonance study of chitin. *Macromolecules, 23,* 3576–3583.
Wada, M., Kwon, G. J., & Nishiyama, Y. (2008). Structure and thermal behavior of a cellulose I–ethylenediamine complex. *Biomacromolecules, 9,* 2898–2904.
Wada, M., Nishiyama, Y., & Langan, P. (2006). X-ray structure of ammonia–cellulose I: New insights into the conversion of cellulose I to cellulose III$_I$. *Macromolecules, 39,* 2947–2952.
Wada, M., Okano, T., & Sugiyama, J. (1997). Synchrotron-radiated X-ray and neutron diffraction study of native cellulose. *Cellulose, 4,* 221–232.