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Crystal transition of paramylon with dehydration and hydration

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ABSTRACT

The structure of paramylon, a highly crystalline (1 → 3)-β-D-glucan material, was analyzed by synchrotron X-ray powder diffraction and solid-state ^{13}C NMR spectroscopy. Native paramylon, the hydrate with high crystallinity, was converted into the anhydrous form by drying, by which the crystallinity decreased and the molecular conformation became partially disordered. The anhydrous readily resumed the hydrate form by immersing in water, but the crystal size and the homogeneity of molecular conformation did not reach those of the native hydrate. Crystal transition between the hydrate and anhydrous by relative humidity (R.H.) changes was monitored by X-ray diffractometry and gravimetry. The dehydration occurred around R.H. 30%, and the re-hydration occurred around R.H. 70%. The changes in weight and unit-cell volume by dehydration allowed determination of the number of water molecules in the hydrate as one per anhydroglucopyranoside residue.

1. Introduction

(1 → 3)-β-D-Glucan is a polysaccharide occurring in many organisms such as fungi, bacteria, algae and annual plants (Stone & Clarke, 1992). It has attracted much attention in the medical field due to its physiological effects such as immunomodulation and anti-cancer activities (Ooi & Liu, 2000). While many (1 → 3)-β-D-glucans have branches by β-(1 → 6) linkages, two organisms are known to produce strictly linear (1 → 3)-β-D-glucan; those are curdlan, the extracellular polysaccharide of *Alcaligenes* bacteria, and paramylon, the disk-like storage granule in *Euglena* cells (Clarke & Stone, 1960; Harada, Misaki, & Saito, 1968).

The structure of (1 → 3)-β-D-glucan has been studied by X-ray diffraction (Chuah, Sarko, Deslandes, & Marchessault, 1983; Deslandes, Marchessault, & Sarko, 1980; Marchessault, Deslandes, Ogawa, & Sundararajan, 1977; Takeda, Yasuoka, Kasai, & Harada, 1978) and solid-state ^{13}C NMR spectroscopy (Fyfe et al., 1984; Pelosi, Bulone, & Heux, 2006; Saito, Tabeta, & Harada, 1981; Saito, Tabeta, Yokoi, & Erata, 1987; Saito, Yokoi, & Yoshioka, 1989), mainly using curdlan as specimen. One reason for the interests in curdlan is its gelation phenomena in aqueous solutions (Harada et al., 1968; Marchessault et al., 1977; Saito et al., 1981, 1989). For crystal structure analysis, however, paramylon has important advantage of unusually high crystallinity as natural macromolecule.

Marchessault et al. (1977) identified two crystal forms of (1 → 3)-β-D-glucan, hydrate and anhydrous form, by X-ray diffraction of oriented fibers of curdlan prepared from dimethylsulfoxide solution. They subsequently proposed the crystal structure models of hydrate and anhydrous based on X-ray diffraction and stereochemical modeling (Chuah et al., 1983; Deslandes et al., 1980). Their model for anhydrous was a right-handed 6/1 triple helices arranged in a hexagonal unit cell with dimensions of a = 14.41 Å and c = 5.87 Å. The space group was $P6_3$, with the asymmetric unit consisting of one glucopyranoside residue, and the unit cell containing six glucose residues (Deslandes et al., 1980). Based on the structure of anhydrous, that of hydrate was determined from combination of fiber and powder X-ray diffraction as: right-handed 6/1 triple helices arranged in a hexagonal unit cell with dimensions of a = 15.56 Å and c = 18.78 Å. This structure belongs to the space group $P1$, with its c-axis three times longer than that of anhydrous, and is close to $P3$ except for the water molecules. Thus the hydrate has lower symmetry than anhydrous, and the unit cell contains 18 glucopyranoside residues and 36 water molecules (Chuah et al., 1983).

These analyses indicated the presence of one water molecules per glucose residue in the hydrate (Chuah et al., 1983). On the other hand, the number of water molecules in hydrate calculated from density measurement was two per glucose residue (Chuah et al., 1983; Marchessault et al., 1977). Chuah et al. (1983) explained this contradiction as follows: While two water molecules per glucose residue are in the crystal lattice, one is located around the O4 and O5 atoms as hydrating water, and the others are distributed randomly in the crystal. This explanation, however, remained speculative, and more precise analysis is necessary for full understanding of the hydrate structure.

For crystal structure analysis, paramylon has a disadvantage of being granules; but its high crystallinity is an alternative

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advantage utilized in several studies (Booy, Chanzy, & Boudet, 1981; Chuah et al., 1983; Kiss, Roberts, Brown, & Triemer, 1988; Marchessault & Deslandes, 1979). In the present study we reexamined the crystal structures of paramylon by synchrotron X-ray powder diffraction, which allows analysis with higher precision, combined with solid-state cross-polarization/magic angle spinning (CP/MAS) ^{13}C NMR.

Besides uncertainty in the crystal structure, there have been contradictory reports about the transition between hydrate and anhydrous forms of paramylon (Kiss et al., 1988; Marchessault & Deslandes, 1979). Marchessault and Deslandes (1979) reported that the anhydrous needs annealing at 140 °C in water to convert to hydrate. On the other hand, Kiss et al. (1988) reported that the dried samples prepared from never-dried paramylon readily converted to hydrate by immersion in water. Therefore we also studied the reversibility between the hydrate and anhydrous forms of paramylon by preparing never-dried, dried and rehydrated samples from cultured *Euglena*. Also the transitions by humidity change were studied by X-ray diffractometry.

2. Experimental

2.1. Cultivation and preparation of paramylon sample

Euglena gracilis E-6 (IAM Culture Collection) was cultured in a medium containing 5 g of pepton, 2 g of yeast extract, 15 g of glucose, and 10 μg of cyanocobalamine in 1 L of water at 28 °C in the dark (Kitaoka, Sasaki, & Taniguchi, 1993). After cultivation for a week, the cells were collected by centrifugation (8000*g*, 5 min), redispersed in water, and sonicated for disrupting the cells. After centrifugation the pellet was dispersed in 0.1 M-pH 6.8 Tris–HCl buffer containing 2% sodium dodecylsulfate, heated to 95 °C for 15 min, and collected by centrifugation (Kiss et al., 1988). The sample was stored as never-dried, or lyophilized and stored over P_2O_5 in a desiccator; the latter was used for re-hydration experiments.

2.2. Synchrotron X-ray powder diffraction

Synchrotron X-ray powder diffraction was carried out at the beam line BL38B1 at SPring-8 (Hyogo, Japan). The sample sealed in a glass capillary was mounted on a goniometer head and irradiated by synchrotron X-ray beam (λ = 1.0 Å) for 120 s. The diffraction pattern was recorded on a flat imaging plate, R-AXIS V, Rigaku, with a specimen-to-IP distance of 170 mm. The distance was calibrated by using Si powder (d = 3.1355 Å). The peak positions of the X-ray diffraction profiles were determined by peak-fitting by non-linear least-square-fitting program for pseudo-Voigt function (Wada, Okano, & Sugiyama, 1997).

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

Solid-state ^{13}C NMR spectra of never-dried, dried, and rehydrated samples of paramylon were obtained on a CMX 300 spectrometer (Chemagnetics) operating at 75.6 MHz. The sample in a 4.0 mm zirconia rotor was spun at 10 kHz in a solid-state probe at the magic angle. All spectra were obtained using ^{1}H NMR 90° pulse lengths of 2.5 μs, with a cross-polarization time of 2.0 ms and 60 kHz CW proton decoupling. A recycle time was 3 s. The spectra were calibrated by adamantane as standard. The rotor was sealed by a Teflon cap to avoid drying of the sample for the never-dried and rehydrated samples. The deconvolution of the spectra was carried out using the non-linear least-square-fitting procedure where a Lorentzian function was applied for each peak.

2.4. Monitoring transition by X-ray diffractometry

X-ray diffractometry in reflection mode was carried out using a diffractometer (Rigaku, RINT2000), with monochromatic Cu Kα radiation (λ = 1.5418 Å), generated at 38 kV and 50 mA by optical slit system: divergence slit = 0.5°; scattering slit = 0.5°; and receiving slit = 0.15 mm. Scanning was performed as: scattering angle, 2θ = 3–35° with 2θ step of 0.05° and accumulation time of 5 s. Control of relative humidity was done by sealing the goniometer with a polyester (Mylar) film to which humidity-controlled nitrogen gas was introduced from a humidity generator (Shinyei SRG-1R) at a flow rate of 1 L min^{-1}.

For the drying process the never-dried paramylon sample was spread on a glass plate and set in the goniometer under R.H. 100%. After standing overnight, the X-ray diffraction was carried out as described above, with humidity increment of 10% for 100–0% with stabilization time of 1 h each. Re-hydration of the dried sample was performed in the goniometer at R.H. 0%. The X-ray diffraction was taken for relative humidities from R.H. 0–100%. The temperature was about 23 °C.

2.5. Water adsorption measurement

Water desorption/adsorption isotherms of paramylon were determined by equilibrating the samples over saturated salt solutions in desiccators at 23 °C. The amount of bound water was estimated by gravimetry.

3. Results and discussion

3.1. Synchrotron X-ray powder diffraction

The X-ray powder diffraction patterns of the never-dried, dried, and rehydrated samples of paramylon were recorded as in Figs. 1 and 2. The native (never-dried) hydrate (Fig. 2a) gave many sharp peaks up to high Q ranges, indicating exceptionally high crystallinity. The nine peaks in Fig. 2a could be indexed according to Chuah et al. (1983). The present data allowed refinement as: a = 15.574 (1) Å, and c = 18.587 (10) Å (Table 1), closely agreeing with Chuah et al.'s values (1983).

Dehydration of the never-dried paramylon caused conversion to anhydrous as indicated by the change in diffraction profile shown in Fig. 2a and b. Compared to the hydrate, the anhydrous peaks in high Q range are broad and reduced. Thus the transition from hydrate to anhydrous was accompanied by certain decrease in crystallinity. The five peaks numbered in Fig. 2b could be indexed according to the hexagonal unit cell (Deslandes et al., 1980), allowing refinement of the unit cell as: a = 14.543 (7) Å, and c = 5.853 (9) Å (Table 1). The profile of the rehydrated samples prepared by immersing the anhydrous into water was almost the same as that of the never-dried samples, but its peak widths were slightly broader (Fig. 2c). Thus the transitions between hydrate and anhydrous is basically reversible as reported by Kiss et al. (1988).

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

Solid-state ^{13}C NMR spectra of the never-dried, dried and rehydrated samples are shown in Fig. 3. The spectra of dried and rehydrated samples are similar to the previously reported spectra (Fig. 3b and c) (Fyfe et al., 1984; Saito et al., 1987, 1989). On the other hand, some signals of the never-dried samples clearly split into more peaks than that of the rehydrated samples, although the peak positions of the two spectra are the same (Fig. 3a and c). This indicates that the conformations of the never-dried and rehydrated samples were the same, but that of rehydrated samples

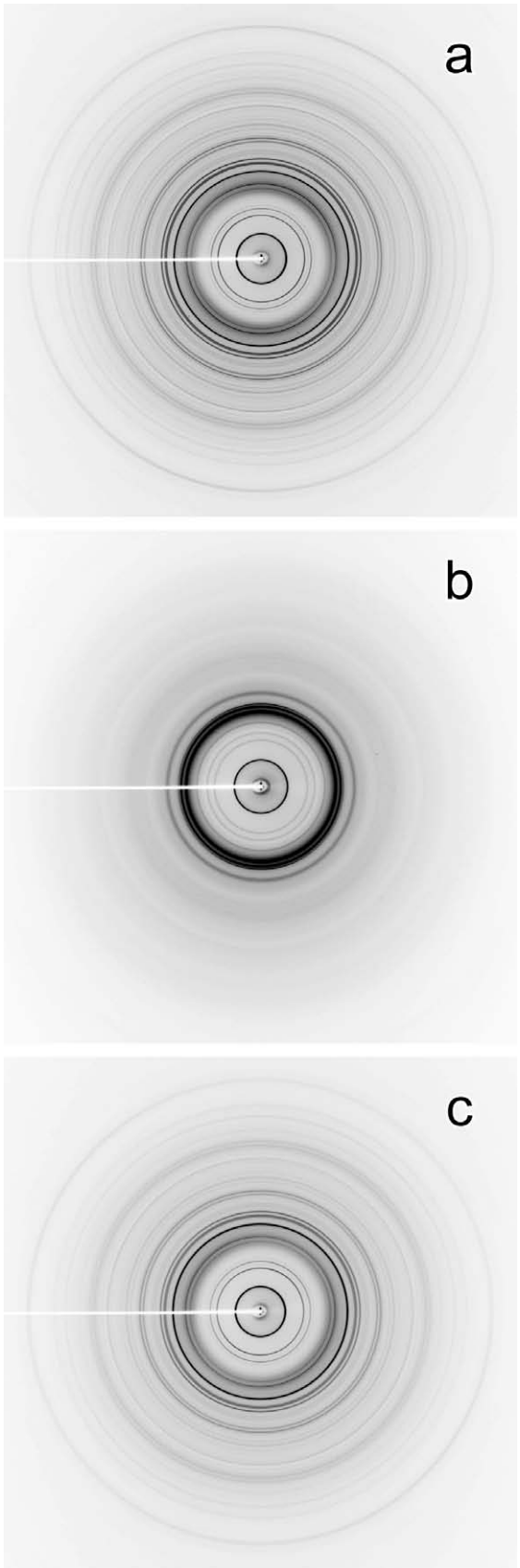

Fig. 1. Synchrotron X-ray powder diffraction diagrams of never-dried (a), dried (b), and rehydrated (c) paramylon samples. Never-dried and rehydrated samples were conditioned under R.H. 85% for eliminating scattering by excess water.

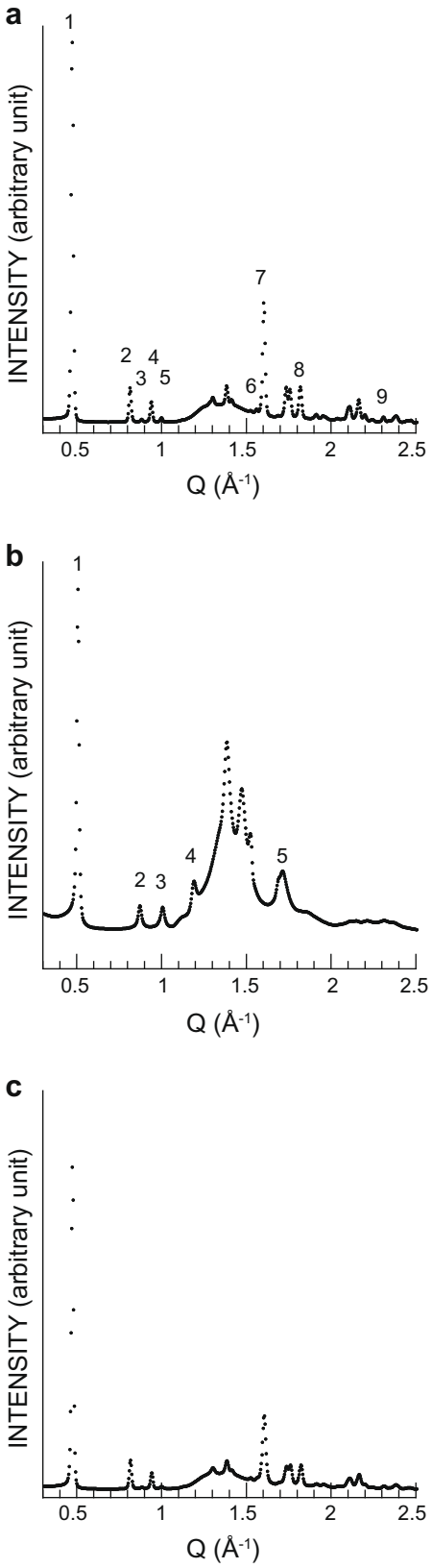

Fig. 2. Synchrotron X-ray powder diffraction profiles of never-dried (a), dried (b), and rehydrated (c) paramylon obtained from intensity integration of the patterns in Fig. 1. Peaks used for unit cell determination are numbered. Q is the scattering vector ($2\pi/d$).

Table 1
Comparison of observed and calculated *d*-spacings for hydrate from never-dried paramylon samples and for anhydrous from dried paramylon sample.

Sample	*h*	*k*	*l*	*d*-Spacings (Å)		
				d_{obs}	d_{cal}	$d_{obs} - d_{cal}$
Never dried[a]	1	0	0	13.489	13.487	0.002
	1	1	0	7.785	7.787	−0.002
	1	1	1	7.181	7.182	−0.001
	2	0	0	6.744	6.744	0.000
	2	0	1	6.339	6.339	0.000
	3	0	2	4.046	4.047	−0.001
	2	1	3	3.937	3.937	0.001
	3	1	2	3.470	3.470	0.000
	4	0	4	2.730	2.729	0.001
Dried[b]	1	0	0	12.599	12.594	0.005
	1	1	0	7.267	7.271	−0.004
	2	0	0	6.296	6.297	−0.001
	1	0	1	5.311	5.308	0.003
	2	1	1	3.683	3.693	−0.010

[a] Calculated *d*-spacings are for a hexagonal unit cell with dimensions a = 15.574 Å and c = 18.587 Å.
[b] Calculated *d*-spacings are for a hexagonal unit cell with dimensions a = 14.543 Å and c = 5.853 Å.

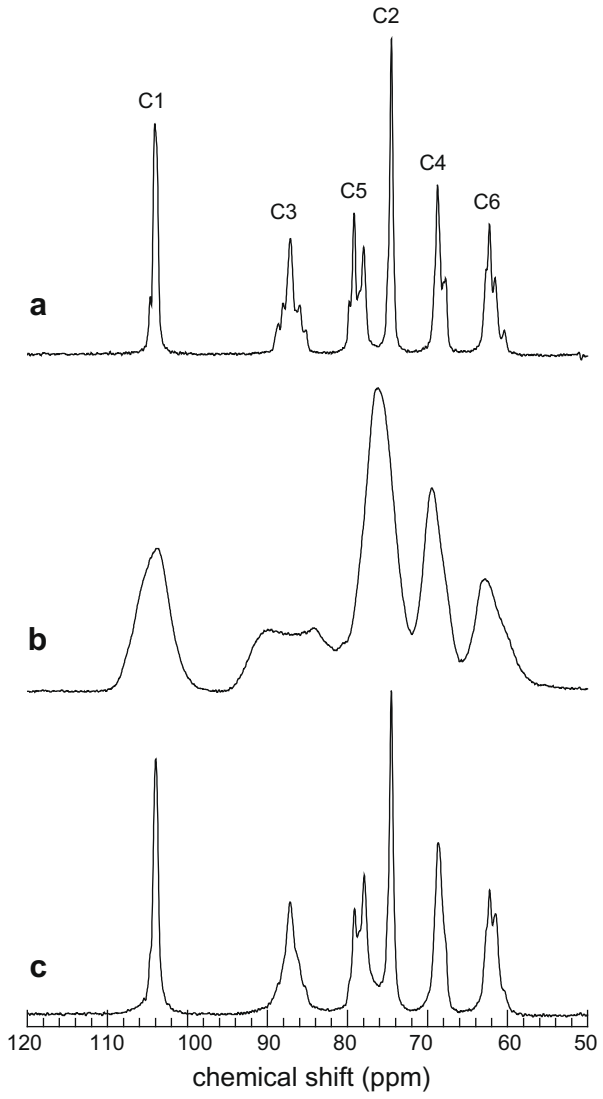

Fig. 3. Solid-state CP/MAS ^{13}C NMR spectra of never-dried (a), dried (b), and rehydrated (c) paramylon. Peak assignments accord to Saito et al. (1989).

became partially disordered through the conversion processes. Together with the X-ray diffraction analysis, these results indicate that the transition between the hydrate and anhydrous is reversible, with slight disruption of the crystalline order and distortion of the molecular chains.

The transition from the hydrate to the anhydrous caused slight shifts in carbon peaks in NMR (Table 2). In addition, all the peaks became much broader (Fig. 3b). Such changes by dehydration are also characteristic of other polysaccharides such as starch and amylose (Horii, Yamamoto, Hirai, & Kitamaru, 1987; Marchessault, Taylor, Fyfe, & Veregin, 1985). The re-hydration resulted in the resumption of peak sharpness (Fig. 3c), indicating recovery of homogeneity in molecular conformations due to release of molecular strain by insertion of water molecules.

The spectrum of the never-dried sample (Fig. 3a) shows splitting of the C3 signal into five peaks; the area of the center peak at 87.2 ppm was about twice as those of other four peaks. This feature indicates that the asymmetric unit of the hydrate must contain six glucose residues. Chuah et al. (1983) reported that the chain conformation is close to the one belonging to space group *P*3, where six glucose residues constitute the asymmetric unit. Thus the present NMR results are consistent with the hydrate model proposed by Chuah et al. (1983).

In the spectrum of the dried sample (Fig. 3b), the C3 signal is splitted into two peaks at 85.2 and 89.9 ppm (Table 2). This is inconsistent with the model of the anhydrous by Deslandes et al. (1980), where the asymmetric unit contains one glucose residue. One of the peaks might be derived from crystalline region and the other from amorphous region. However, Saito et al. (1987) reported that the relaxation time (T_{1c}) of those two peaks was almost the same: 26 and 34 s; therefore it is impossible to assign one of them to amorphous region. Further study is required on this point.

3.3. Monitoring the transition between the hydrate and anhydrous

The hydrate-anhydrous transition was monitored by X-ray diffractometry with stepwise changes of relative humidity at 23 °C as shown in Fig. 4. The original pattern of hydrate (Fig. 4a, R.H. 100%) contains many sharp peaks; with decrease in relative humidity these peaks gradually shifted to higher *Q* ranges, but the overall pattern remained the same until R.H. 40%. At relative humidity of 30%, a conspicuous change took place, apparently by dehydration, which seems to complete at R.H. 20%. In the wetting process (Fig. 4b) each peak gradually shifted to lower *Q* sides, and at R.H. 70%, the peaks became broader, and the hydrate pattern resumed completely at R.H. 80%. Subsequently, the peaks became sharper as R.H. increased. These results clearly show that the hydrate-anhydrous transition is reversible, but with a significant hysteresis.

Table 2
^{13}C chemical shifts for never-dried and dried paramylon samples.

Sample	Chemical shifts (ppm)					
	C1	C2	C3	C4	C5	C6
Never dried	103.8	74.5	85.2	67.7	78.0	60.4
	104.1		86.0	68.0	78.5	61.5
	104.7		87.2	68.7	79.2	62.3
			88.1		79.8	62.7
			88.8			
Dried	104.2	76.1[a]	85.2[b]	69.3	76.1[a]	62.2
			89.9[b]			

[a] Peak at 76 ppm was assigned to both C2 and C5 atoms.
[b] Peak spread around 80–90 ppm was assigned to C3, and separated into two peaks.

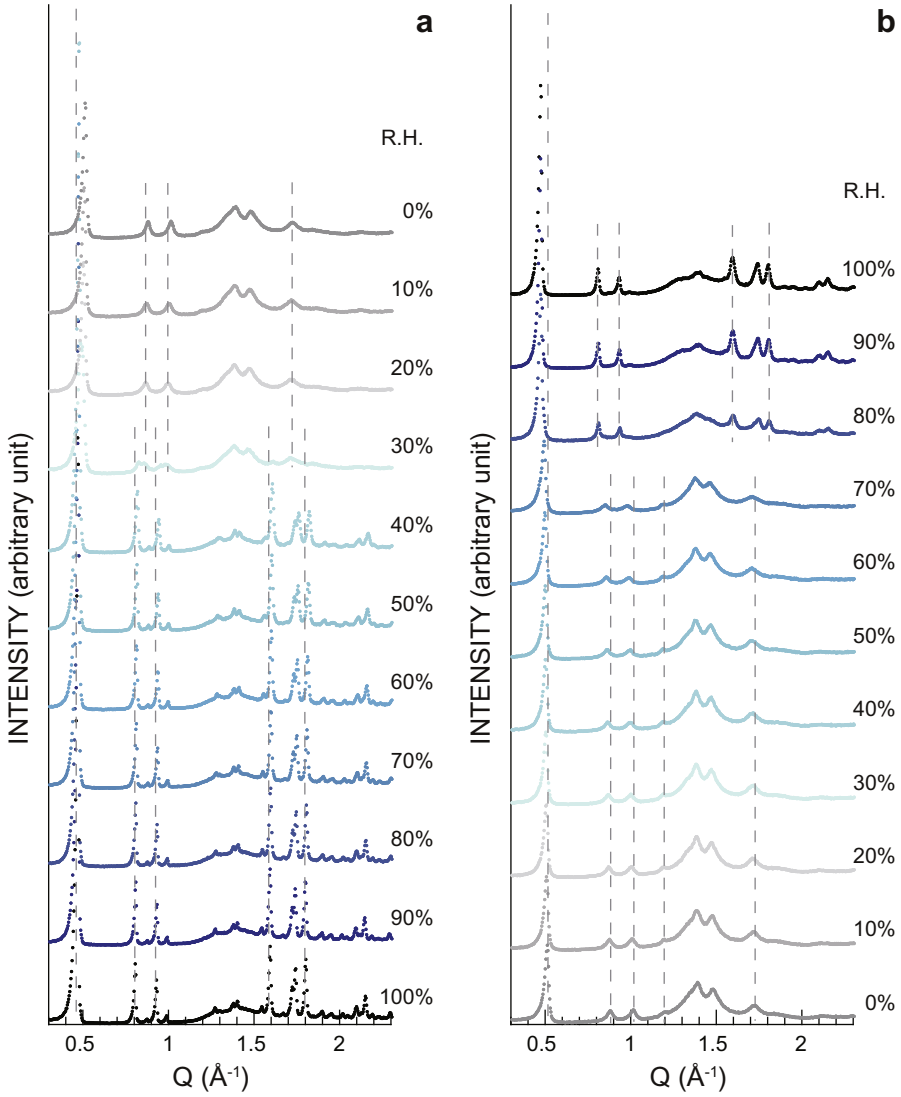

Fig. 4. Change in X-ray diffraction profile during drying of native paramylon (a) and during wetting of dried paramylon (b). Q is the scattering vector ($2\pi/d$).

For understanding the changes in the crystal structure during the drying and wetting processes, the unit-cell parameters at various R.H. were calculated based on the hexagonal unit cell of hydrate and anhydrous. The changes in the unit-cell parameters and volume are shown in Fig. 5. In this figure the length of *c*-axis and unit-cell volume of anhydrous are tripled for comparison with hydrate. The values around the transition points are not shown because of the difficulty in separation of overlapping peaks from the two forms. During both the drying and the wetting processes, the *a*-axis gradually changed even before reaching the transition points. The difference in *a*-axis for R.H. 100% and 0% was approximately 1.2 Å, but the differences just before and after the transitions were approx. 0.8 Å. On the other hand, the *c*-axis was constant except for the transition regions. The change in unit-cell volume was similar to that of the *a*-axis; i.e. steep changes in transition regions and gradual changes in other regions.

3.4. Gravimetric isotherm and stoichiometry

Fig. 6 shows the water desorption/adsorption isotherms of paramylon determined by gravimetry. The overall pattern corresponds closely to that of Fig. 5, showing distinct hysteresis between adsorption and desorption. The transitions R.H. were approximately 30% and 70% in the dehydration and re-hydration, respectively, also agreeing with the values from X-ray diffractometry.

The weight difference between R.H. 95% and 0% was more than 20% (dry base). This amount, however, must contain adsorbed water outside the crystal lattice; therefore the amount of hydration water was determined from the weight change around the transition points as shown in Fig. 6. The resulting value was approximately 12% (dry base) for both drying and wetting processes (Fig. 6). This value corresponds to one water molecule per one glucopyranoside residue. On the other hand, the differences in the unit-cell volume from diffractometry were approximately 600 $Å^3$ (Fig. 5). Multiplying this value with the density of ice, 0.92 g/cm^3, the number of water molecules in the unit cell is estimated as 18.5. Thus the most likely stoichiometric value is 18, i.e. one water molecule per glucopyranoside.

Because of their limited size, paramylon crystallites must have certain surface that can adsorb water. Therefore, the gradual weight changes outside the crystal transition regions (Fig. 6) can be ascribed to the adsorbed water. The corresponding changes in the *a*-axis and the unit-cell volume (Fig. 5) may result from relaxation or plasticization of the crystallites due to adsorption of water.

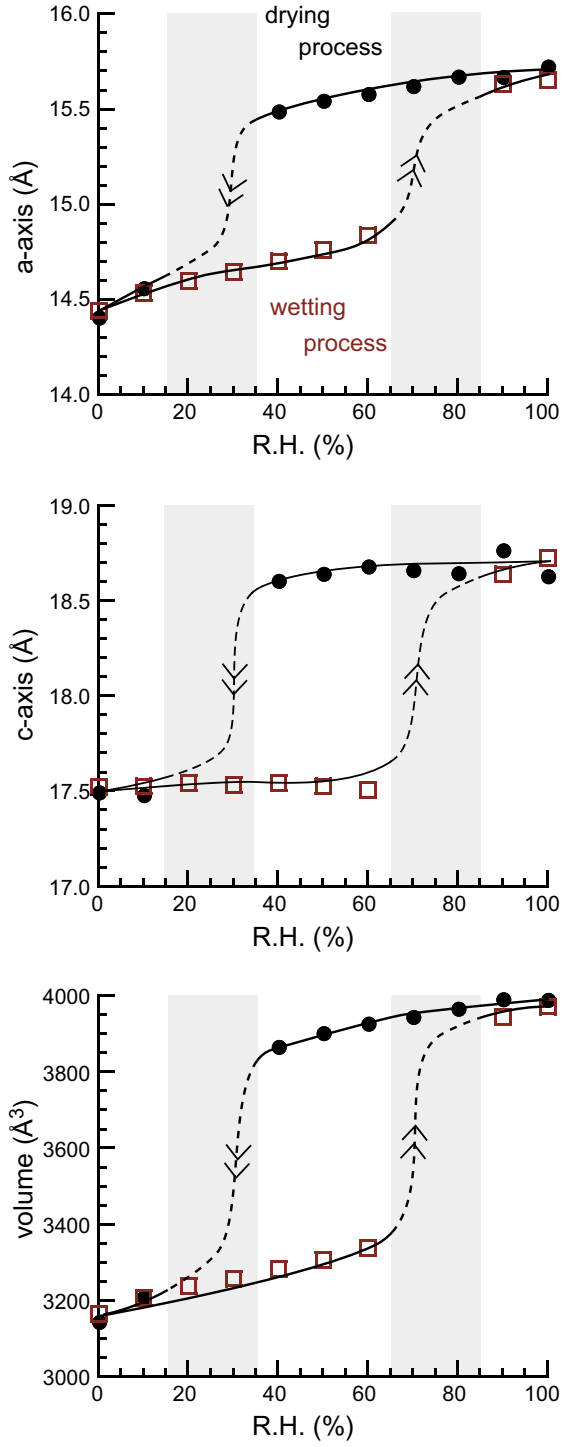

Fig. 5. Changes in unit-cell parameters calculated from Fig. 4 for drying (filled circles) and wetting (open squares). The gray areas show transition regions. *c*-axis and unit-cell volume of anhydrous are tripled for making correspondence with those of hydrate.

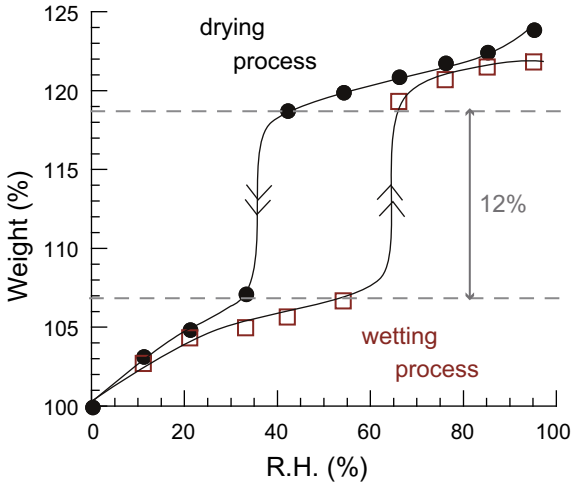

Fig. 6. Water desorption/adsorption isotherms of paramylon.

4. Conclusion

The never-dried, dried, and rehydrated paramylon samples were prepared from *Euglena* cultured in the dark. Synchrotron X-ray powder diffraction, solid-state CP/MAS ^{13}C NMR spectroscopy, and gravimetry analyses of the crystal structure change by hydration/dehydration lead to the following conclusions:

1. The transitions between the hydrate and anhydrous take place reversibly. The anhydration by drying is accompanied by significant decrease in crystallinity, possibly resulting from introduction of molecular distortion.
2. The transition shows a large hysteresis for humidity changes; the transition from hydrate to anhydrous occurs at around R.H. 30%, and the reverse transition at around R.H. 70%.
3. Humidity changes outside the transition regions cause adsorption/desorption of water on the crystallite surfaces, which affects the crystal structures to some degrees.
4. The hydrate contains one water molecule per one glucopyranoside residue.

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