Molecular and Crystal Structure of Hydrated Chitosan

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ABSTRACT: The molecular and crystal structure of the hydrated form of chitosan, which was obtained by deacetylation of chitin from crab tendon, was determined by the X-ray fiber diffraction method and the linked-atom least-squares method. The chitosan chains crystallize in an orthorhombic unit cell with dimensions $a = 8.95(4)$, $b = 16.97(6)$, c (fiber axis) $= 10.34(4)$ Å and a space group $P2_12_12_1$. The chain conformation is a 2-fold helix stabilized by O3---O5 hydrogen bond with the *gt* orientation of O6. The unit cell contains four chains and eight water molecules. There are direct hydrogen bonds (N2---O6) between adjacent chains along the *b*-axis, which makes a sheet structure parallel to the *bc*-plane. These sheets stack along the *a*-axis. Each sheet is related to its neighboring sheet by 2_1-symmetry along the *b*-axis. Water molecules form columns between these sheets and contribute to stabilize the structure by making water-bridges between polymer chains.

Introduction

Chitosan, the N-deacetylated chitin, has received much attention as a functional biopolymer for diverse applications due to its remarkable ability to form specific complexes with a number of ions including transition and posttransition metal ions.[1] Although several structural studies of the chitosan complexes with metal salts have been reported so far, none of the structures were analyzed because of their complexity. Therefore, before analyzing chitosan complexes with metals, we focused our attention on the structure of hydrated chitosan from which chitosan complexes are formed.

The X-ray fiber diffraction data of chitosan was first derived from the solid-state deacetylated product of a crab tendon chitin.[2] The tendon chitosan thus obtained shows a hydrated crystalline form which can be converted to an anhydrated form by annealing.[3] The crystal structure of chitosan anhydrated form was analyzed recently.[4] In this structure, polymer chains take a 2-fold helical conformation with the fiber repeating unit of 10.34 Å, which is very popular among $\beta(1\rightarrow4)$ linked polyglucose, polymannose, and polyglucosamine. Two such chains are packed in an antiparallel fashion in the $P2_12_12_1$ orthorhombic unit cell. Adjacent parallel chains are connected by the O6---N2 hydrogen bond, which makes a sheet structure along the *b*-axis. No hydrogen bonds were observed between these sheets along the *a*-axis. Similar packing was also proposed by electron diffraction study of a single crystal of chitosan anhydrated form.[5] On the other hand, the structure of the chitosan hydrate is yet unknown, although some structural information was obtained from renatured chitosan film specimens.[6] Since the deacetylation of chitin, transformation from the hydrated to the anhydrated form of chitosan, and chitosan−metal complex formation occur in the solid-state, the structure of hydrated chitosan is very important to clarify these solid-state transformations. Furthermore, chitosan makes acid salts and complexes with transition metal ions in the solid-state only when it is crystallized in a hydrated form. Therefore, the structural elucidation of hydrated chitosan is essential for the clarification of these salt and complex formations.

The present study provides a precise crystal structure of hydrated chitosan from the tendon chitin by means of the X-ray fiber diffraction method and the linked-atom procedure. For the intensity data collection and structure analysis, we used the in-house software system in which an imaging plate was used as an X-ray detector.[7,8]

Materials and Methods

Preparation of Specimens. A tendon chitosan was prepared from the chitin of a crab tendon, *Chionoecetes opilio* O. Fabricius, by N-deacetylation with 50% sodium hydroxide solution at 110 °C for 2 h under nitrogen atmosphere, after overnight removal of inorganic compounds with dilute HCl solution. The deacetylation procedure was repeated twice to get complete conversion. The degree of N-acetylation by colloidal titration and the viscosity average degree of polymerization of the tendon chitosan thus obtained were found to be 0% and 10 800, respectively.[9]

X-ray Diffraction Measurement. The X-ray diffraction patterns were recorded by a camera system equipped with an imaging plate (DIP-100S, MAC Science Co. Ltd.) by using graphite-monochromatized Cu Kα radiation (1.5418 Å) from an X-ray generator (Rotaflex RU-200, Rigaku Co. Ltd.). The Mo Kα radiation (0.7107 Å) was also used to get higher angle data on the equator and the meridian for space group determination. During X-ray diffraction measurement, the specimen fiber was kept in the sample holder with 100% RH by flowing wet He gas.

The X-ray image was read by measuring the fluorescent intensity stimulated by a focused He−Ne laser beam scanning the surface of the imaging plate (IP). After converting into pixel data in a rectangular coordinate system, several correction factors, such as a geometric factor and a uniformity factor,

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were applied. The X-ray image was stored as 5 MB of pixel data. Each diffraction spot was manually picked by positioning the center of the diffraction spots with a mouse and coordinates were measured (SUN SP/2, A SUN Microsystems Computer Corporation Business). After determining the center and the inclination angle of the X-ray pattern, the interplanar spacing was obtained by averaging the distances between the center of the diffraction pattern and the positions of two or four equivalent reflections. The unit cell dimensions were determined by a least-squares method with the preliminary cell dimensions obtained by a trial-and-error method on the computer display.

The removal of background intensity was carried out for each diffraction spot as follows. First, the diffraction spot was enclosed by a fanlike shape defined by two radii and one angle at the circumference. The background intensity was estimated from the volume under the surface defined by four average values. Each value was the average of the five lowest intensities in one of four subareas at the corners within the above enclosed area. Second, the intensity distribution of the diffraction spot along the axial and angular directions was assumed by a pseudo-Voigt function, which was a linear combination of a Gaussian and a Lorentzian. The shape of the intensity peak was defined by specifing boundaries of its profile defined by a pseudo-Voigt function along the axial and angular directions. Depending on boundaries, the peak was encircled by any shape between a circle and a crescent. Integrated intensity was obtained by accumulating intensities at all pixels within this enclosed area.[7,10]

The measured intensities (I_o) were corrected for the Lorentz and polarization factors. The absorption effect was not corrected in this study. The Lorentz factor, L, is given by the following equation when there is no fiber tilting to the normal to the X-ray beam.[11]

$$\frac{1}{L} = 2\pi \sin\theta(\cos^2\theta - \cos^2\sigma)^{1/2}$$

in which $\tan\sigma = \zeta/\xi$. The polarization factor, p, is given by the following equation when the primary beam is partially polarized by a monochromator crystal.[12]

$$\frac{1}{p} = \frac{(\cos^2 2\theta_M \cos^2\rho + \sin^2\rho)\cos^2 2\theta + \cos^2 2\theta_M \sin^2\rho + \cos^2\rho}{1 + \cos^2 2\theta_M}$$

Here, θ is the Bragg angle of the reflection, θ_M is the Bragg angle of the 002 reflection plane of the carbon graphite monochromator ($\theta_M = 13.29°$ for Cu Kα and $\theta_M = 6.09°$ for Mo Kα), (ξ, ζ) are the cylindrical polar coordinates in reciprocal space, and $\cos\rho = \zeta/\sin 2\theta$ when both the diffraction plane of the monochromator and the fiber axis are at a horizontal setting. Then, the observed structure factor, F_o, will obtained by

$$F_o = (I_o/Lp)^{1/2}$$

The reflections below the observational threshold were also included in the structure analysis. That is, unobserved reflections with longer spacings than that of the most outside reflection on the same layer line were assumed to have half of the intensity of the observational threshold. These unobserved reflection data were used only when the magnitude of the calculated structure amplitude became larger than that of the estimated structure amplitude ($|F_c| > |F_{unobs}|$) during refinement.

Thermal Analysis. To remove water trapped in the microvoid of the tendon specimen, the specimen was immersed in the acetone solution and degassed to replace air in the void by acetone. To remove acetone in the microvoid completely, the specimen was kept in a vacuum desiccator at 50 °C for 30 min. The chitosan specimen thus obtained was examined by thermogravimetry as well as differential thermal calorimetry (THERMO PLUS TG 8110, Rigaku Co.) at a scanning rate of 10 °C min^{-1} from room temperature to 300 °C under nitrogen atmosphere. The specimen just before thermogravimetry was also submitted to X-ray diffraction, which showed exactly the same diffraction pattern as the hydrated chitosan specimen.

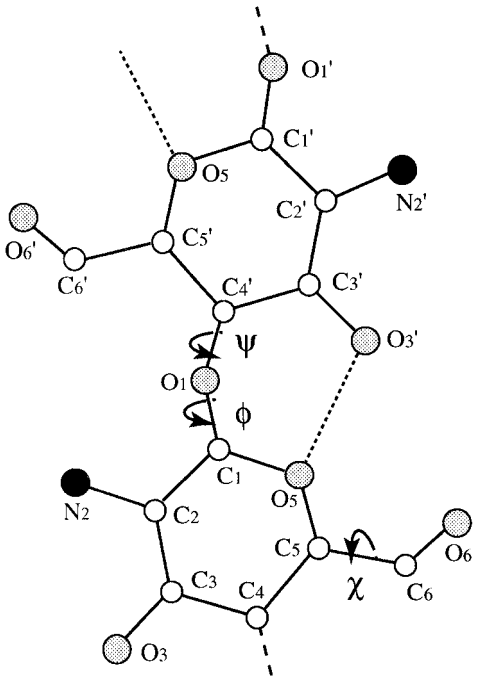

Figure 1. Chemical structure of chitosan together with atomic numbering. Dashed lines denote O3---O5 hydrogen bonds. Two dihedral angles (ϕ, ψ) defining the main chain conformation and one dihedral angle χ defining the O6 orientation are also shown.

Density Measurements. Density was measured by the flotation method with a solution of trichloromethane and dichloroethane. Before the measurement, it was necessary to degas from the microvoid in the specimen.

Molecular Model Building. Molecular models having the 2/1-helical symmetry and the fiber repeating period of 10.34 Å together with the pyranose ring in the standard $^4C_1$ chair conformation were generated by using a linked-atom description[13] with fixed bond lengths and angles.[14] Although there are two dihedral angles in a chemical repeating unit to define the main-chain conformation, there is no degree of freedom in the main chain conformation under the constraining conditions of the observed unit height (10.34 Å/2) and unit twist (360°/2). The dihedral angles at the glycosidic linkage, ϕ and φ, were fixed to the values that satisfy these conditions. At the final stage of the analysis, however, these were refined together with the glycosidic linkage angle. In addition to ϕ and φ, there is a dihedral angle (χ) to define the orientation of the O6 oxygen atom (Figure 1). This was assumed to be one of three values of ±60° or 180° at the first stage, since many related compounds revealed that this angle fell into one of these: *gauche*−*gauche* ($\chi = -60°$), *gauche*−*trans* ($\chi = 60°$), and *trans*−*gauche* ($\chi = 180°$).[15]

Packing Models and Their Refinement. To obtain appropriate packing models, the fractional coordinates (u, v, w) of the origin atom (C1) and the azimuthal angle (μ) have to be determined. Here, u and v are the positional parameters of polymer chain in the *ab*-plane, w is the height of the origin along the *c*-direction, and μ is the azimuthal angle of the polymer around the molecular axis. In the first step, the positional parameters u and v and the azimuthal angle μ were searched in terms of R-values for the equatorial reflections. The packing models obtained were further investigated to get the appropriate positional parameter, w, by using all the reflections including those unobserved. From this stage, the O6 atom was attached to the C6 atom with one of three orientations of *gg*, *gt*, and *tg*. In the second step, two water molecules were manually introduced per asymmetric unit by

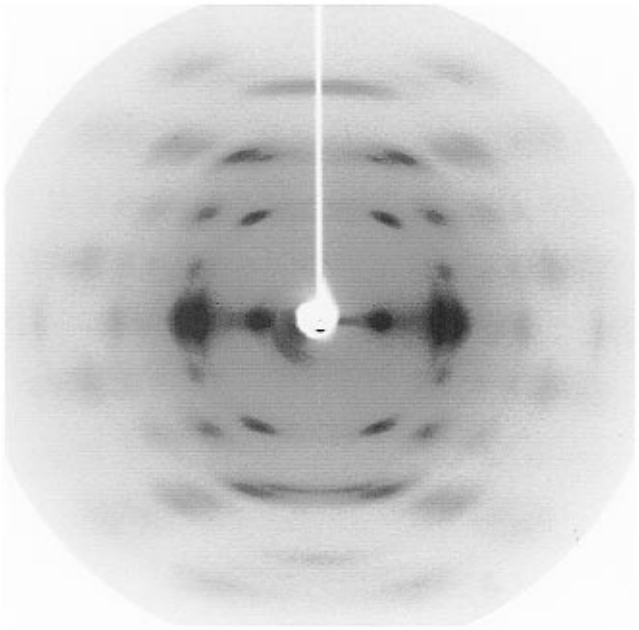

Figure 2. X-ray diffraction pattern of hydrated chitosan recorded on the imaging plate.

a systematic search. As the last step, plausible packing models with different locations and occupancies of water molecules were considered.

At each stage of the modeling and refinement, the quantity Ω was minimized in the following least-squares fashion.[13]

$$\Omega = \sum w(|F_o| - |F_c|)^2 + s\sum \epsilon_j + \sum \lambda_h G_h$$

The first term ensures the optimum agreement between the observed (F_o) and the calculated (F_c) X-ray structure amplitudes. The weight of the reflection, w, was fixed to 1.0 for observed reflections and 0.1 for unobserved reflections. The second ensures the optimization of noncovalent interatomic interactions. The third imposes, by the method of Lagrange undetermined multipliers, the exact constraints we have chosen. The agreement between observed and calculated structure amplitudes was evaluated by R and R_w, which were defined by

$$R = \sum ||F_o| - |F_c||/\sum |F_o|, \quad R_w = \sum w(|F_o| - |F_c|)^2/\sum wF_o^2$$

Atomic scattering factors for calculating structure factors were obtained by using the method and values given in the literature.[16] Computations were done on a SUN workstation (SUN SP/LX, A SUN Microsystems Computer Corporation Business).

Structure Determination

Crystal Data. Figure 2 shows an X-ray diffraction pattern of the hydrated chitosan recorded on an imaging plate. A total of 24 observed diffraction spots were indexed by a rectangular unit cell with dimensions a = 8.95(4), b = 16.97(6), c (fiber axis) = 10.34(4) Å. In the X-ray diffraction pattern taken by Mo Kα radiation, systematic absences of odd reflections on h 0 0, 0 k 0, and 0 0 l were observed up to 7 0 0, 0 11 0, and 0 0 5, respectively. Therefore, the space group was determined as $P2_12_12_1$.

The thermogravimetric (TG) measurement of tendon chitosan showed a 10.7% weight decrease at 250 °C compared to the weight at room temperature (Figure 3), which suggests that the specimen contains two water molecules in an asymmetric unit (1 H_2O/monosaccharide).

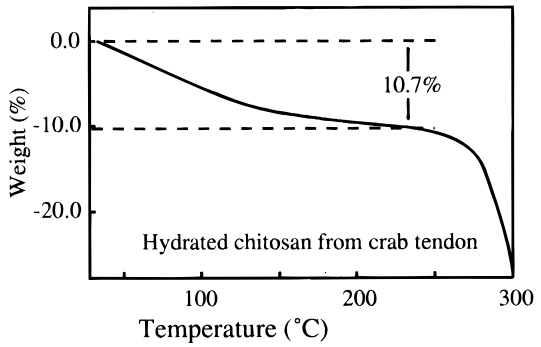

Figure 3. Thermogravimetric measurement of tendon chitosan showing 10.7% weight loss below 250 °C.

Table 1. Packing Analysis of Hydrated Chitosan

packing model	R_w after refinement		packing model with $2H_2O$/asym	R_w after refinement
UD*gg*	0.28	→	UD*gg*	0.26
UD*tg*	0.31			
UD*gt*	0.32			
UU*gg*	0.28	→	UU*gg*	0.25
UU*tg* }	0.28	→	UU*gt* 12	0.21
UU*gt* }			UU*gt* 13 }	0.23
			UU*gt* 23 }	
GP*gg* }	0.36			
GP*tg* }				
GP*gt*	0.34	→	GP*gt*	0.27

The number of chemical repeating units of the polymer chain in the unit cell was assumed to be eight from the observed density (ρ_o = 1.45) and the unit cell volume. By structural analogy with other β(1→4) linked polysaccharides such as cellulose and chitin, we decided that the chitosan molecule has a 2/1-helical symmetry within the repeating period of 10.34 Å. From the observed density, the plausible number of water molecules in a unit cell was also deduced. If four or eight water molecules are included in a unit cell, the calculated density is 1.44 or 1.52, respectively. Taking the water content and the observed density into account, the number of water molecules in a unit cell seems to be eight, that is, two water molecules in an asymmetric unit (one for each saccharide residue). This was also supported by the X-ray agreement between $|F_o|$ and $|F_c|$ in the last stage of analysis.

Molecular and Crystal Structure. There are two ways to pack four polymer chains with 2/1-helical symmetry in a unit cell with the space group $P2_12_12_1$. One is to locate the polymer chains at special positions, that is, on the crystallographic 2_1-axes at (u = 0.25, v = 0.0) and (u = 0.75, v = 0.0). The other is to locate the chains in the general positions. In the former case, there are two independent polymer chains. Therefore, we must investigate two possible cases about polymer direction. That is, two independent polymer chains are in the same direction or in the opposite directions. We call these packing models UU and UD. Here, UU or UD stands for two chains pointing in the same direction (two up-pointing) or opposite directions (one up- and one down-pointing), respectively. In both cases, molecular azimuthal angles μ_1 and μ_2 were around 100° ($\mu_1 = \mu_2$ = 100° for the UU model and μ_1 = 100°, μ_2 = 80° for the UD model) from the X-ray agreement (R-values) for the equatorial reflections.

In the general position case, the chain position (u, v) and the azimuthal angle μ were searched within the asymmetric unit by calculating R-values for the equatorial reflections. As a result, three appropriate positions,

Table 2. UU*gt* Packing Models with Different Water Schemes

packing model	occupancy of water				including unobs		excluding unobs			
	W1	W2	W3	$\Omega \times 10^{-5}$	R_w	R	R_w	R	attn	shortest contact
UU*gt*12	1	1	0	1.81	0.20	0.22	0.19	0.18	12.7	2.39Å Ow---O3
UU*gt*13	1	0	1	2.11	0.20	0.22	0.20	0.18	11.1	2.32Å Ow---H6
UU*gt*123	1	$^1/_2$	$^1/_2$	2.47	0.21	0.23	0.20	0.18	9.9	1.95Å H1---H5
UU*gt*2/3	$^2/_3$	$^2/_3$	$^2/_3$	2.44	0.21	0.23	0.20	0.18	11.2	1.96Å H1---H5
UU*gt*3/3	1	1	1	3.37	0.22	0.24	0.21	0.19	15.7	1.90Å H1---H5

($u = v = 0.0$), ($u = 0.0$, $v = 0.25$), and ($u = v = 0.25$) with the same azimuthal angle ($\mu = 100°$) were obtained. We call these packing models GP, GP1, and GP2, respectively. GP stands for general position. As a result, there were five packing models, UU, UD, GP, GP1, and GP2. So far, to make the molecular conformation simple, the orientation of the hydroxymethyl moiety was not considered by excluding the O6 atom from the calculation.

In the next step, we searched the positional parameter (w) of the polymer chain along the c-direction for each packing model with one of three orientations of hydroxymethyl moiety. Now, the number of packing models was 15 (=5 × 3). These were distinguished by the *gg*, *tg*, and *gt* following the notation of UU, UD, GP, GP1, and GP2. Each of the 15 models was refined against all the reflections, including unobserved ones. At this stage, all the GP1 and GP2 models had high R-values (more than 0.48) and these were excluded from further analyses. The rest of them are listed in Table 1. Since the orientation of the hydroxymethyl moiety of UU*tg* and GP*gg* became close to those of UU*gt* and GP*tg*, respectively, a total of seven were subjected to further analyses. In each case, one water molecule was systematically located at one of the grid points in the asymmetric unit and the R-factor was calculated. By comparing R-factors at every positions, the appropriate water position for each model was investigated. Although R-factors of four, UD*gg*, UU*gg*, UU*gt*, and Gp*gt*, decreased by about 5%, no such decrease was observed for UD*tg*, UD*gt*, and GP*tg*. Therefore, the latter were not considered for further analysis.

For the four models (UD*gg*, UU*gg*, UU*gt*, and GP*gt*), the second water molecule was introduced. In the case of GP*gt* and UD*gg*, there was no decrease in R-factor by addition of a second water molecule. On the other hand, in the case of UU*gt*, three water locations were found. Therefore, we considered three models, UU*gt*12, UU*gt*13, and UU*gt*23, which have different combinations of water locations; the integer denotes the location of water molecules. In the UU*gt*23 case, the water molecule at position 2 became close to position 1 of UU*gt*13 after several refinement cycles. In the case of UU*gg*, the R-factor was higher than for the UU*gt* models and one short contact was also found between water oxygen and H6 atoms. Therefore, only UU*gt*12 and UU*gt*13 were subjected to further consideration.

Since we found three water locations which were stereochemically acceptable, the occupancy at each location was studied. Table 2 shows R- and Ω-values of packing models with different locations and occupancies, including three additional models. UUgt123 has a water molecule with full occupancy at position 1 (W1) and two more with half-occupancies at positions 2 (W2) and 3 (W3). UUgt2/3 and UUgt3/3 models have three water molecules with two-thirds and full occupancy at each of the three positions, respectively. Except for the last model, the number of water molecules in an asymmetric unit is two. In the last model, one additional water molecule could not help in the improvement of the R-value and it caused an increase in the attenuation factor (attn). On the other hand, addition of up to two water molecules improved the R-values. Therefore, the models with two water molecules were considered more appropriate, even though three water molecules could be accommodated without any steric hindrance. Two water molecules are consistent with density measurement and thermogravimetric analysis.

Table 3. Final Parameters of Hydrated Chitosan

Torsion Angles and Bond Angle at Glycosidic Linkage, deg	
ϕ(C2−C1−O1−C4')	145.9
φ(C1−O1−C4'−C3')	94.1
v(C1−O1−C4')	114.2
Orientation of O6, deg	
θ(O5-C5-C6-O6)	68.6
Packing Parameters of Polymer Chains	
μ1, deg	96.4
w1	0.3905
μ2, deg	84.8
w2	0.1368
Locations of Water Molecules	
W1 (u3, v3, w3)	(−0.0671, 0.2482, 0.2990)
W2 (u4, v4, w4)	(0.0616, 0.3057, −0.1349)
W3 (u5, v5, w5)	(0.0766, 0.2262, 0.5352)
scale factor	0.9367
attn	11.2
R	0.21
R_w	0.23
R(ex)	0.20
R_w(ex)	0.21

Table 4. Fractional Atomic Coordinates[a] of Hydrated Chitosan

	X	Y	Z
O1	0.3228	0.0358	1.0045
C1	0.2454	0.0218	0.8904
O5	0.2838	−0.0558	0.8481
C5	0.2094	−0.0769	0.7300
C6	0.2479	−0.1622	0.7028
O6	0.1815	−0.2141	0.7950
C2	0.2886	0.0851	0.7929
N2	0.2417	0.1601	0.8396
C3	0.2193	0.0678	0.6616
O3	0.2734	0.1232	0.5686
C4	0.2581	−0.0156	0.6186
H1	0.1234	0.0255	0.9027
H2	0.4111	0.0872	0.7835
H3	0.0971	0.0741	0.6679
H4	0.3785	−0.0193	0.5976
H5	0.0877	−0.0729	0.7436
H6a	0.3644	−0.1697	0.7067
H6b	0.2085	−0.1770	0.6104
HN1	0.2809	0.1804	0.9237
HN2	0.1694	0.1924	0.7882
OW1[b]	−0.0671	0.2482	0.2990
OW2[b]	0.0616	0.3057	−0.1349
OW3[b]	0.0766	0.2262	0.5352

[a] The fractional atomic coordinates (X_N, Y_N, Z_N) of another independent chain will be generated by the following equations from the above coodinates (Xo, Yo, Zo). $X_N = (Xo - 0.25)\cos\theta + Yo\sin\theta + 0.75$. $Y_N = (Xo - 0.25)\sin\theta + Yo\cos\theta$. $Z_N = Zo - w_1 + w_2$. Here, $\theta = \mu_1 - \mu_2$. [b] The occupancy for these water oxygen atoms is $^2/_3$.

Table 5. Observed (F_o) and Calculated (F_c) Structure Amplitudes of Hydrated Chitosan[a]

reflcn no.	*h*	*k*	*l*	F_c	F_o	reflcn no.	*h*	*k*	*l*	F_c	F_o
1	0	2	0	110.7	123.7	38	1	7	1	63.4	53.4
	1	1	0			39	0	1	2	7.9	(8.4)
2	1	2	0	54.8	(14.1)	40	1	0	2	18.9	(12.2)
3	1	3	0	14.1	(15.9)	41	0	2	2	59.7	117.1
4	2	0	0	335.6	301.7		1	1	2		
	2	1	0			42	1	2	2	39.0	(14.1)
	0	4	0			43	0	3	2	19.0	(15.0)
5	2	2	0	210.9	218.3	44	1	3	2	91.7	103.1
	1	4	0				2	0	2		
6	2	3	0	50.8	(19.7)	45	2	1	2	10.9	(17.8)
7	1	5	0	53.3	46.9	46	0	4	2	4.9	(17.8)
	2	4	0			47	2	2	2	21.9	(17.8)
8	3	1	0	75.7	70.3	48	1	4	2	49.5	(18.7)
	0	6	0			49	2	3	2	41.9	64.7
	3	2	0				0	5	2		
9	2	5	0	9.4	(22.5)	50	1	5	2	17.7	(20.6)
10	1	6	0	8.7	(22.5)	51	2	4	2	11.4	(20.6)
11	3	3	0	56.5	(22.5)	52	3	0	2	30.0	36.5
12	3	4	0	0.8	(23.4)		3	1	2		
13	2	6	0	29.4	(23.4)		0	6	2		
14	1	7	0	9.2	(24.4)	53	1	0	3	18.4	(12.2)
15	3	5	0	94.0	75.9	54	0	2	3	62.3	76.8
	4	0	0				1	1	3		
	4	1	0			55	1	2	3	62.0	110.6
16	0	1	1	0.2	(8.4)	56	0	3	3	14.7	(15.0)
17	1	0	1	20.5	(12.2)	57	1	3	3	27.3	(15.9)
18	0	2	1	10.6	(12.2)	58	2	0	3	74.2	80.6
19	1	1	1	23.0	(12.2)		2	1	3		
20	1	2	1	11.9	(14.1)		0	4	3		
21	0	3	1	9.5	(15.0)	59	2	2	3	88.0	88.1
22	1	3	1	31.6	34.7		1	4	3		
23	2	0	1	46.7	71.2	60	2	3	3	36.1	(19.7)
	2	1	1			61	0	5	3	45.1	(19.7)
	0	4	1			62	1	5	3	72.1	61.8
24	2	2	1	11.0	(17.8)		2	4	3		
25	1	4	1	25.7	(18.7)		3	0	3		
26	2	3	1	35.1	45.0		3	1	3		
	0	5	1			63	0	6	3	77.1	60.0
27	1	5	1	34.6	(20.6)		3	2	3		
28	2	4	1	42.9	(20.6)		2	5	3		
29	3	0	1	54.0	60.0		1	6	3		
	3	1	1			64	1	2	4	14.9	(14.1)
30	0	6	1	16.9	(21.6)	65	0	3	4	38.7	58.1
31	3	2	1	22.5	(21.6)	66	1	3	4	22.2	(15.9)
32	2	5	1	33.3	(22.5)	67	2	0	4	79.1	100.3
33	1	6	1	27.6	(22.5)		2	1	4		
34	3	3	1	57.4	76.8		0	4	4		
35	3	4	1	65.1	(23.4)		2	2	4		
36	0	7	1	69.0	(23.4)		1	4	4		
37	2	6	1	12.2	(23.4)						

[a] Reflections with F_o values in parentheses are those for unobserved reflections. These values are half of the observational threshold.

From these experimental results, we decided that most of the asymmetric units contain two water molecules located at two of three water positions, W1, W2, and W3. Since there are no significant differences among the four models, we took the UU*gt*2/3 model as a hydrated chitosan structure. Of course, the polymer conformation and the polymer packing in these four models are essentially the same, the difference among them comes only from water locations and occupancies.

Final values of refined parameters are listed in Table 3 and the fractional coordinates are given in Table 4. Observed and calculated structure amplitudes are listed in Table 5. The X-ray agreement factors R and R_w are 0.21 and 0.23 for reflection data including the unobserved one, and 0.18 and 0.20 for 24 observed reflections, respectively. The packing structures are shown in Figure 4, in which hydrogen bonds are denoted by dashed lines.

Result and Discussion

Comparison of Unit Cell Parameters with Those in the Previous Works. In the pioneering work of Clark and Smith,[2] the orthorhombic unit cell with cell dimensions of $a = 8.9$, $b = 17.0$, and c (fiber axis) $= 10.25$ Å was proposed for tendon chitosan from lobster tendon. These are very similar to the values obtained in this study. On the other hand, in the model proposed by Sakurai et al.[6] the unit cell ($a = 8.67$, b (fiber axis) $= 10.24$ and $c = 8.92$ Å and $\beta = 92.6°$) is one-half of our unit cell. In their study, regenerated films from chitosan powder was used as a sample. Although the intensity distribution in the fiber diffraction pattern was very similar, and most of the reflections found in this study could be indexed by their unit cell, several reflections on the first layer line could not be indexed. Together with the number of reflection data, obviously better crystallinity and orientation of tendon chitosan used in this study suggested better reliability of unit cell parameters compared with those for the regenerated chitosan film.

Molecular Structure. Chitosan molecules in the hydrated form have a 2-fold helical symmetry reinforced by the O3---O5 hydrogen bond with a repeating period of 10.34 Å; this is a typical structure for the $\beta(1\rightarrow4)$

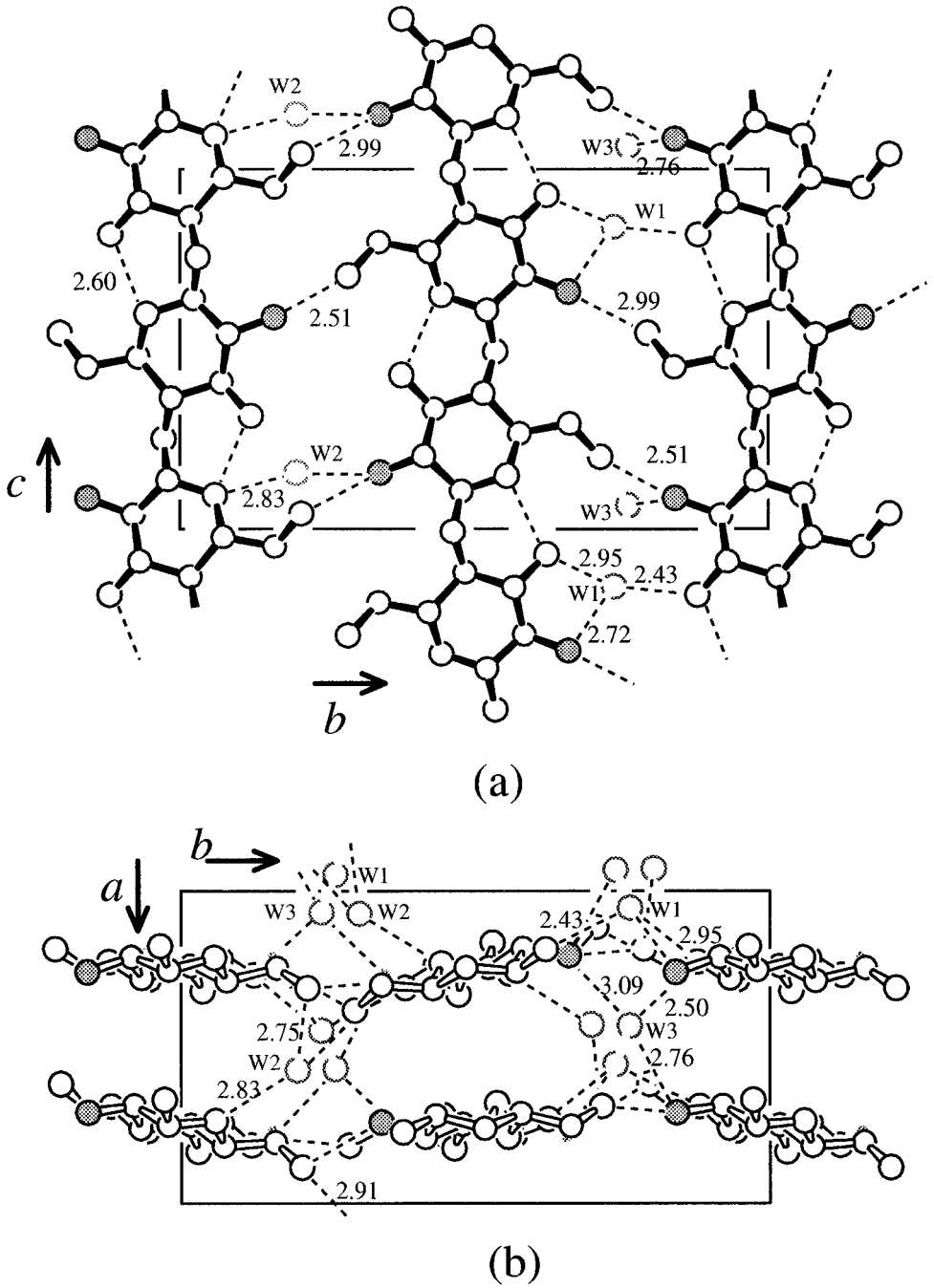

Figure 4. Packing structure[19] of hydrated chitosan projected along the *a*-axis (a) and along the *c*-axis (b). Filled circles denote nitrogen atoms. For the sake of clarity, only three polymer chains of the lower layer in (b) are shown in (a).

linked polysaccharides such as cellulose, mannan, and chitin. The main-chain conformation angles, ϕ(C2−C1−O1−C4′) and φ(C1−O1−C4′−C3′), are 145.9° and 94.1°, respectively. The orientation of O6 has a *gt* conformation (χ(O5−C5−C6−O6) = 68.6°). According to the Cambridge Structural Database,[17] out of 161 examples in oligosaccharide crystals, 109 cases take *gg*, 51 *gt*, and only one *tg* conformations. Although *gt* is the second most common conformation in the single crystal structures of oligosaccharides, this is found in many polysaccharide structures including the anhydrated form of chitosan.

Crystal Structure. Two adjacent polymer chains along the *b*-axis are crystallographically independent (Figure 4). These two chains are arranged in an antiparallel fashion and are linked to each other by two N2---O6 hydrogen bonds (2.51 and 2.99 Å) along the *b*-axis, which makes the sheet structure parallel to the *bc*-plane (Figure 4a). These sheets are piled up along the *a*-direction (Figure 4b). Neighboring sheets are related by crystallographic 2_1-symmetry along the *b*-direction. As a result, two independent polymer chains with the same direction form a repeating unit along the *a*-axis. These two chains have a translational difference along the *c*-axis of approximately one quarter of the fiber repeating unit (Table 3). This quarter-staggered structure was consistent with the intensity distribution on the meridian, notably the very weak 002 and strong 004 reflections. A similar quater-staggered structure was also reported in cellulose I.[18] There are no direct interactions between sheets, but several hydrogen bonds via water molecules are observed, which stabilize the packing structure. These are W2---O5 (2.83), W2---O6 (2.75 and 2.91), W3---O3 (2.50), and W3---N2 (3.09 and

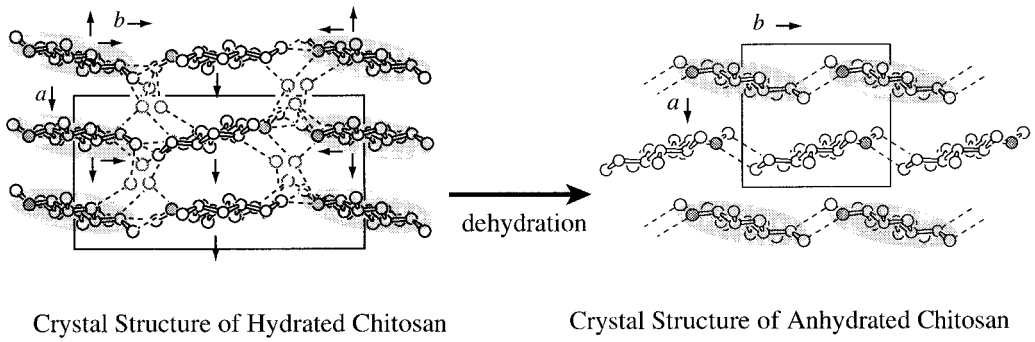

Figure 5. Plausible solid-state transformation from the hydrated form to the anhydrated form of tendon chitosan. Shaded polymer chains denote up-pointing molecules. Hydrogen bonds before and after transformation are also shown.

2.76 Å). On the other hand, W1 connects adjacent molecules in the same sheet by hydrogen bonds W1---O3 (2.95 and 2.43) and W1---N2 (2.72 Å).

Although there are three water locations, X-ray agreement, observed density, and thermogravimetric analysis match with two water molecules in an asymmetric unit. Furthermore, no significant differences were found between the positions of the two waters in UU*gt*12 and UU*gt*13 and the three waters in UU*gt*123 and UU*gt*2/3. Therefore, it is unknown why the asymmetric unit contains only two water molecules even though there are three appropriate positions. This rather loose crystal structure of hydrated chitosan may facilitate the complex formation with transition metals in the solid phase.

Crystal Transformation from the Hydrated to the Anhydrated Form. The simplest conversion process from the hydrated to the anhydrated form in the solid-state can be explained as follows. When dehydration occurs, to fill the space where two water molecules exist, all the down-pointing molecules (unshaded molecules in the hydrated structure in Figure 5) shift 0.25 along the *a*-axis by breaking the hydrogen bonds between neighboring polymer chains. At the same time, the shrinkage along *b* and the enlargement along *a* take place to form the alternate layer structure of up-pointing and down-pointing chains in the anhydrated structure. During this transformation, two independent neighboring molecules along *a* in the hydrated form must become equivalent in the anhydrated form. Therefore, one of two independent chains must shifts along the *c*-axis by about *c*/4. Since there is no direct evidence for the crystal transformation process, the structure analyses of chitosan complexes with transition metal salts, which can be formed only from the hydrated chitosan, may help to understand the conversion process better.

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