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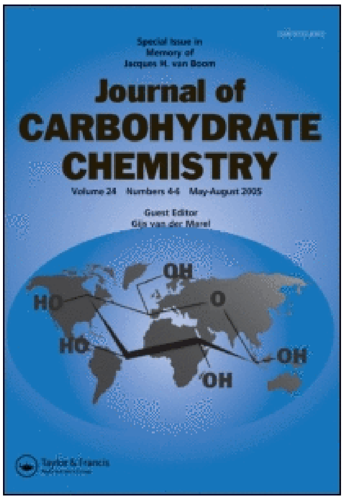

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SINGLE-HELICAL STRUCTURE OF NATIVE CURDLAN AND ITS AGGREGATION STATE[1]

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ABSTRACT

One of the polymorphs of β-(1→3)glucan, curdlan, was studied by X-ray diffraction and thermal analyses. Different from the molecular models proposed so far, the molecular structure of native curdlan (Form I) was found to be a 6/1-single stranded helix. Therefore, the transformation from Form I to Form II is a conversion process from single to triple. Form I specimen has three kinds of water, bound- and amorphous-water in the crystalline micelles and free water in the intermicelles. The amount of bound water is estimated to be more than 10 %wt. If the specimen is kept at less than 98%RH, the effusion of free water and amorphous water occurs very easily, which causes a drastic decrease of the specimen weight. The bound water is left even at 0%RH.

INTRODUCTION

Curdlan is an extracellular, bacterial polysaccharide composed of (1→3)β-linked D-glucose residues. It is produced from the mutant strain of the bacteria Alcaligenes faecalis var. myxogenes 10C3. This polysaccharide was first isolated and investigated by Harada and coworkers[2] who named it "curdlan" to describe the gelling behavior at elevated temperatures. This polymer has several interesting physicochemical features, such as the ability to form a firm and resilient gel when heated in aqueous suspension.

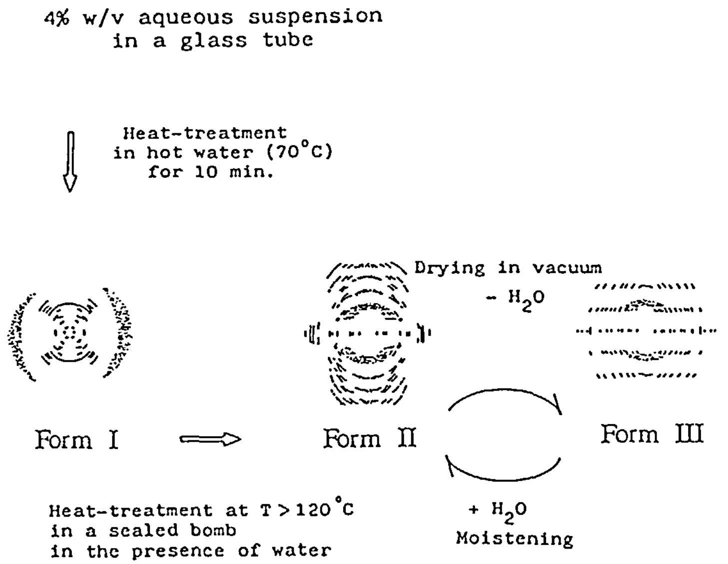

FIG.1. Structural changes between three forms of curdlan and schematic illustrations of their X-ray fiber diagrams.

A number of X-ray diffraction patterns of oriented curdlan gel have been reported.[3-7] These are classified into three crystalline modifications, Form I, Form II and Form III (Fig.1). Form I is prepared from a 4% aqueous suspension of curdlan powder by heat-treatment in hot water at about 70 °C for 10 min. Form II is prepared from Form I by heat-treatment above 120 °C in a sealed bomb in the presence of water. This is transformed to Form III by drying in vacuum. The difference between Form II and III is the existence of water in the crystal and these two modifications can be transformed mutually by the control of relative humidity. Form II and III allomorphs are highly crystalline and their structures were shown as a triplex composed of right-handed 6/1 helices.[4,5] On the other hand, Form I (native form) is usually a low crystalline allomorph and its structure has not been clear so far. Based on their X-ray diffraction data from Form I, Kasai and coworkers proposed the mixture model of 7/1-single and 7/1-triple helix,[6] while Atkins and coworkers proposed a 7/1-triple helix model.[7] In this paper we reinvestigate the structure of Form I molecule and discuss the highly hydrated

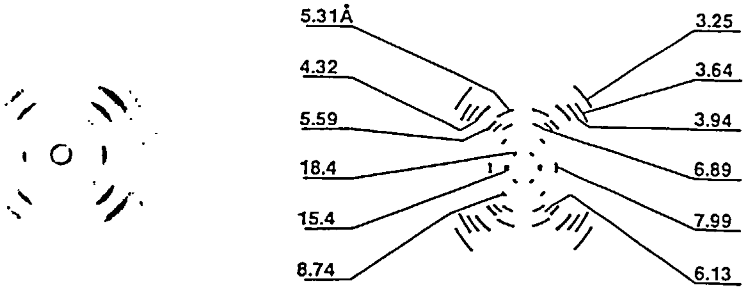

FIG.2. X-ray diffraction pattern taken by a cylindrical camera and its schematic illustration.

aggregation state of Form I to obtain a better understanding of curdlan.

RESULT AND DISCUSSION

Molecular and Crystal Structure of Native Curdlan. The X-ray diffraction pattern from Form I (native form) taken at 98%RH is shown in Fig.2, together with its schematic illustration. All the reflections in the pattern could be indexed by the rectangular unit cell with the lattice parameters of a=28.8, b=16.6 and c (fiber axis)=22.8 Å. With the exception of two reflections (one with the spacing of 18.4Å on the first layer line and the other with 6.13Å on the 3rd layer line), reflections could be also indexed with the hexagonal unit cell similar to those proposed by Atkins et al.[7] Therefore, considering the small number of X-ray data and diffuse intensity distribution, the unit cell parameters used in this analysis are essentially the same as those reported in the previous studies [6,7](Table 1). Since there were not enough reflection data to determine the space group, P1 was used in the following calculations.

Since the right-handed helical sense in Form II and III, which are obtained from Form I by a solid-state transformation, had been established[4,5], only right-handed models were considered. Further, in Form II and III crystals, all the chains are packed in a parallel

TABLE 1. Crystal Data of Curdlan Form I.

	This work	Kasai et al.[6]	Atkins et al.[6]
a/Å	28.8	26.4	17.01
b/Å	16.6	16.4	17.01
c(fiber axis)/Å	22.8	22.65	22.70
Crystal system	ortho.	ortho.	hexagonal
Sample condition	98 %RH	WET	100 %RH
Density/gcm^{-3}	1.27	1.17	1.24
Proposed model	6/1-single	7/1-single + 7/1-triple	7/1-triple

fashion, therefore, only the parallel packing models were considered for Form I. Various right-handed models, such as 5/1-, 6/1-, 7/1-, 8/1- and 9/1-helix for single-, double- and triple-stranded models, were investigated by using a linked-atom least-squares procedure.[8] Among these, the 6/1-single helix model (Fig.3) was the most preferable in terms of interatomic contacts and agreement between observed and calculated structure amplitudes (R=0.135 and Rw=0.179, here $R= \Sigma||Fo| - |Fc|| / \Sigma Fo$ and $Rw=\Sigma|Fo^2 - Fc^2| / \Sigma Fo^2$). The final packing parameters μ_1, μ_2 and w were 24.0°, 163.5° and 0.103, respectively. The final atomic coordinates and the comparison of calculated and observed structure amplitudes are listed in Tables 2 and 3. The only one variable dihedral angle, $\angle O_6C_6C_5O_5$, indicated a trans conformation (-175.4°).

The 6/1-helical model was also supported by the finding of the meridional reflection on the 6-th layer line in the diffraction pattern taken by inclining the specimen about 12°. All the double- and triple-stranded models, with the exception of 5/1-helix, were plausible in terms of stereochemistry. These models were, however, excluded by their poor agreement between observed and calculated structure amplitudes.

The following two experimental observations also support the above single-stranded structure for Form I. One is the difference in preparation conditions between Form I and II. The preparation of Form II needs more energy than that of Form I (Fig.1). Since the structure of Form II has been established as a triple-strand,[4] the transformation from Form I to Form II must be a process from a single-strand to a

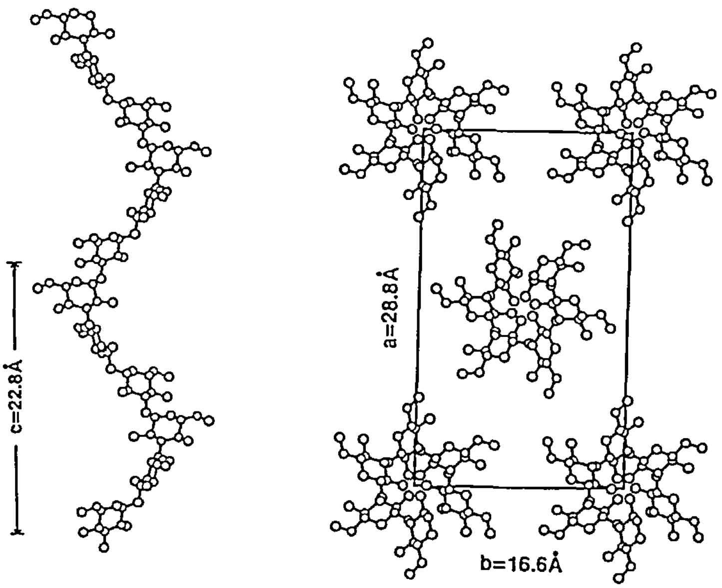

FIG.3. Molecular and crystal structures of curdlan Form I.

triple-strand. The other is that the thermal degradation temperature of Form I is different from that of Form II. The overall appearances of the thermal degradation curves of Form I and II are very similar to each other (Fig.4). The degradation temperature of Form I, however, is lower than that of Form II by about 30 ^{o}C. This difference must be due to the molecular structure difference between Form I and II. Since Form II is a triple-stranded structure, it is very reasonable to think that Form I is a single-stranded structure.

The molecular conformation of Form I with 6/1-helical symmetry is stabilized by the intramolecular hydrogen bond between O_4 and O_5 (dis($O_4 \ldots O_5$)=3.14 Å and $\angle(C_4\text{-}O_4 \ldots O_5)$= 97^{o}). Like the hydrogen bond between O_3 and O_5 in $\beta(1\rightarrow4)$glucan, such as cellulose, mannan and chitin, the O4---O5 hydrogen bond stabilizes the $\beta(1\rightarrow3)$ glycosidic linkage. Two polysaccharide molecules are located at the corner and the center of the unit cell (Fig.3). As shown in later, the unit cell also contains water molecules between polysaccharide chains accounting for more than 70 %wt (about 250 water molecules). There are no direct interactions between adjacent chains.

TABLE 2. Cartesian Coordinates of the Final Structure.[a]

atom	x/Å	y/Å	z/Å
C_1	2.519	1.123	11.410
C_2	2.192	0.842	9.949
C_3	3.063	-0.283	9.414
C_4	4.581	0.041	9.711
C_5	4.759	0.350	11.194
C_6	6.174	0.760	11.540
O_1	1.798	2.240	11.814
O_2	0.813	0.515	9.831
O_4	5.391	-1.084	9.381
O_5	3.903	1.437	11.579
O_6	7.129	-0.206	11.102
H_1	2.278	0.225	11.999
H_2	2.362	1.752	9.354
H_3	2.791	-1.227	9.911
H_4	4.916	0.903	9.114
H_5	4.509	-0.542	11.787
H_{6a}	6.268	0.869	12.630
H_{6b}	6.407	1.719	11.054

a. The coordinates of the other molecule in the unit cell can be generated from these values by the translation (u, v, w) = (14.4, 8.3, 2.339) and the rotation $\mu = 139.8^{\circ}$.

Microcrystalline Structure of Native Curdlan. In order to get a better understanding about Form I specimen, the state of water in the specimen was investigated. Figure 5 shows the melting curves of ice in the Form I specimens kept at various relative humidities for more than one week. For reference, the melting curve of ice itself is also shown in this figure. Judging from the resemblance of heat absorption peaks at 0 °C to that of ice itself, three specimens kept at more than 95%RH seem to have free water. On the other hand, the specimens kept under drier conditions (92%RH and 95%RH) seem to have no such free water. In order to confirm these results, the absolute water contents and densities were measured for the specimens kept at various relative

TABLE 3. Comparison of Caluculated and Observed Structure Amplitudes.

h k l	h k l	Fc	Fo	h k l	h k l	Fc	Fo
1 0 0		0.02	23.9^a	±5 0 4		28.6	61.5^a
0 1 0	2 0 0			0±3 4		49.7	62.3^a
±1 1 0		153.4	147.1	±4 2 4	±4-2 4		
±2 1 0		9.9	39.0^a	±5 1 4	±5-1 4		
3 0 0		6.4	41.6^a	±1 3 4	±1-3 4	128.3	109.5
±3 1 0	0 2 0			±2 3 4	±2-3 4	26.6	63.9^a
±1 2 0		78.9	102.2	±6 0 4	±3 3 4		
				±3-3 4	±6 1 4		
±1 0 1		66.3	54.6	±6-1 4	±5 2 4		
				±5-2 4		119.7	116.4
±1 0 2		28.6	35.3^a				
±1 1 2	±1-1 2			±1 0 5		10.1	53.8^a
0±1 2	±2 0 2	80.3	61.9	0±1 5		10.5	54.9^a
				±2 0 5	±1 1 5		
±1 0 3		6.4	42.0^a	±1-1 5		46.7	55.5^a
0±1 3	±2 0 3			±2 1 5	±2-1 5	21.3	57.1^a
±1 1 3	±1-1 3	63.5	65.5	±3 0 5		10.1	58.1^a
±2 1 3	±2-1 3	41.2	69.7	0±2 5	±3 1 5		
±3 0 3		47.0	47.7^a	±3-1 5		42.4	59.5^a
±1 2 3	±1-2 3			±1 2 5	±1-2 5	43.9	59.9^a
0±2 3	±3 1 3			±2 2 5	±2-2 5		
±3-1 3		131.0	133.4	±4 0 5		8.2	61.3^a
				±4 1 5	±4-1 5	56.8	61.0^a
±1 0 4		6.2	48.1^a	±3 2 5	±3-2 5	66.0	61.8^a
±2 1 4	±2-1 4			±5 0 5		38.1	64.8^a
±1 1 4	±1-1 4			0±3 5		37.6	65.6^a
0±1 4	±2 0 4	37.1	84.3	±1 3 5	±1-3 5		
±3 0 4		11.7	53.6^a	±4 2 5	±4-2 5		
0±2 4	±3 1 4			±5 1 5	±5-1 5	55.9	62.0^a
±3-1 4		59.0	55.3^a	±2 3 5	±2-3 5	65.6	67.0^a
±1 2 4	±1-2 4	36.9	55.8^a	±6 0 5	±3 3 5		
±2 2 4	±2-2 4			±3-3 5	±5 2 5		
±4 0 4	±4 1 4			±5-2 5	±6 1 5		
±4-1 4		142.3	145.9	±6-1 5		117.6	124.6
±3 2 4	±3-2 4	35.7	59.7^a				

^{a}Unobserved reflection

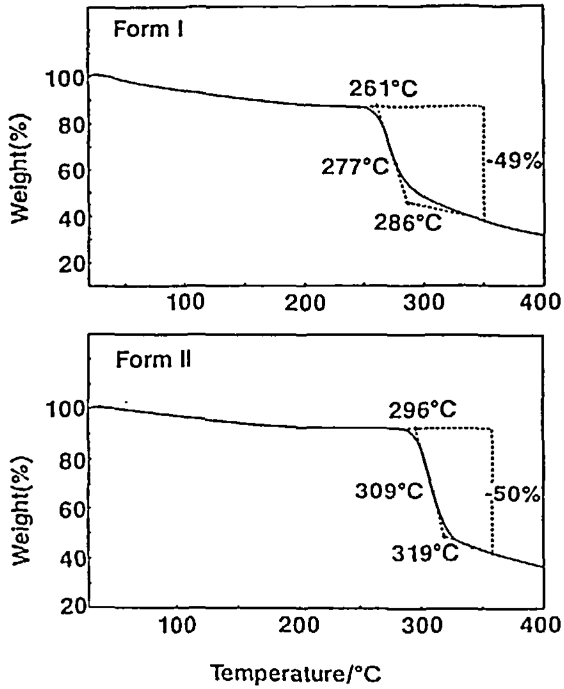

FIG.4. Thermal degradation curves of Form I (above) and Form II (below).

humidities (Fig.6). Up to 92%RH, observed densities gradually increased with the decrease of relative humidity. After that point, the density showed an almost constant value (=1.47). The same tendency was observed for water content. That is, up to 92%RH, water content drastically decrease from 90% to 10% by weight with a small decrease of relative humidity. After that, the water content is almost constant (10%wt). From the analysis of the small angle X-ray scattering along the equator, Kasai et al.[6] proposed that the ultrastructure of Form I specimen consists of crystalline micelles with a diameter of 80Å and water in the intermicelles (Fig.7). Taking into account the facts observed in this study together with the above ultrastructure of Form I, it is concluded that at high relative humidity state the specimen contains three kinds of water, free water in the intermicelles, amorphous water and crystalline bound water in the crystalline micelles.

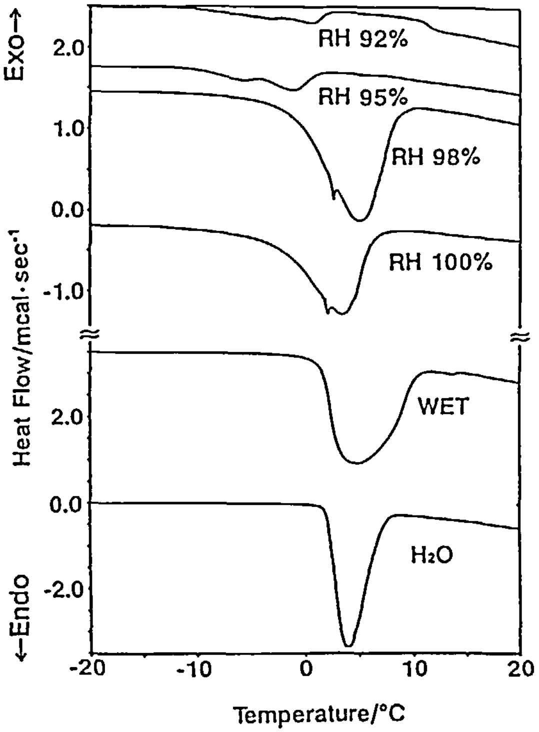

FIG.5. DSC curves of curdlan Form I kept at various relative humidities together with melting curve of ice (bottom).

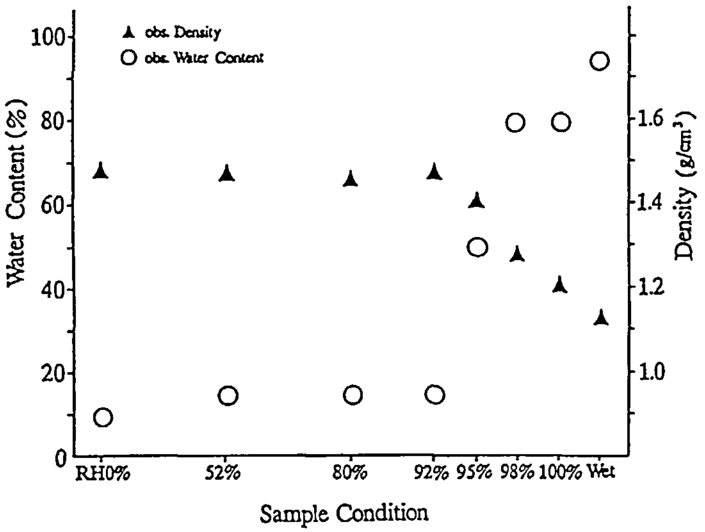

FIG.6. Observed densities and water contents of curdlan Form I kept at various relative humidities.

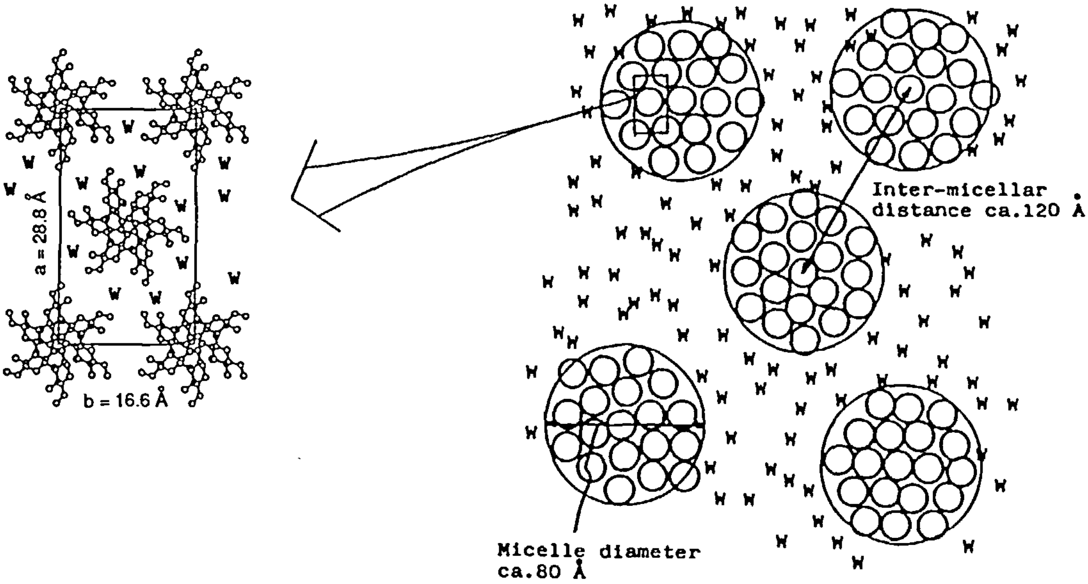

Fig.7. Schematic illustration of an ultrastructure of curdlan Form I. W represents a water molecule.

In conclusion, as shown in Fig.7, an ultrastructure of Form I specimen consists of crystalline micelles and free water between them. The micelle contains several unit cells, which consist of polysaccharide chains and a fair amount of water. A part of this water is crystalline bound water tightly linked to the polysaccharide chains. The amount of bound water is more than 10%wt from Fig.6. Another part of water is amorphous water in the unit cell. Like free water, it is removed easily from the specimen when kept under 95%RH. If there is no water in a unit cell, the calculated density of the crystalline part is only 0.3. Therefore, the unit cell must contain more than 70%wt of water. Since the amount of bound water is around 10%wt, that of amorphous water may be estimated to be 60%wt. Now, it is obvious that the drastic decrease of water content in Fig.6 corresponds to the effusion of free water in intermicelles and amorphous water in micelles and the constant value corresponds to the amount of bound water in the unit cell.

EXPERIMENTAL

Sample Preparation. An aqueous suspension (4% w/v) of commercially available curdlan powder (Wako Pure Chemical Ind., Japan) was sealed in a glass tube and heated in hot water about 70°C for 10 min. Obtained curdlan gel was first dried at room temperature and kept at various relative humidities more than one week.

Density was measured by a flotation method with benzene and carbon tetrachloride.

X-ray Diffraction. X-ray diffraction diagrams were recorded with a cylindrical camera using Ni-filtered Cu $K\alpha$ radiation from a microfocus X-ray generator (ROTA FLEX RU-200, Rigaku Corp.) The intensities (Io) were estimated by visual comparison with a standard intensity scale. These were corrected for the Lorentz and polarization factors (Lp) but not for absorption effects. A total of 12 observed and 28 below threshold diffractions were used in the following calculations. The intensity of the latter diffractions were assumed to be one-half of the observed weakest intensity and used in the calculation only when $|Fo| < |Fc|$.

Structure Analysis Molecular models having the appropriate helical symmetry and the fiber repeating period, and pyranose rings in the standard $^4C_1$ chair conformation[9], were generated using a linked-atom description with all bond lengths and angles held constant.[8] Since two variable parameters at the glycosidic linkage, ϕ and ψ are constrained by the fiber repeating period and the helical symmetry, there is no degree of freedom in the main chain conformation (in the case of a 6/1-helix, $\phi = \angle C_2C_1O_1C_3 = 168.6^\circ$ and $\psi = \angle C_1O_1C_3C_2 = -113.2^\circ$). For the molecular conformation, therefore, the dihedral angle $\angle O_6C_6C_5O_5$ is the only variable parameter. In the following calculations, the initial value of this angle was set to one of -60°, 60° and 180°. When the polymer is located in the unit cell, three parameters have to be determined. These are the azimuthal angles μ_1 and μ_2 for the corner and center molecules and the difference of the relative position along the fiber axis, w. The packing models were generated systematically by changing these μ and w at 30° and 0.04 intervals, respectively. Out of 120 models, 11 models, which were found to be suitable in terms of interatomic contacts and agreement between observed and calculated structure amplitudes, were refined further. Out of 11 models, 6 models converged to the same structure (R=0.14) and the rest of the models had rather poor X-ray agreements (R=0.18-0.22). Therefore, the former structure was refined further.

Thermal Analysis. For thermal analyses, THERMOFLEX DSC 8230 and TG 8110 (Rigaku Co.) were used with a scan speed 10°C/min.

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