Artificial Chitin Spherulites Composed of Single Crystalline Ribbons of α-Chitin via Enzymatic Polymerization

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ABSTRACT: A crystalline product of artificial chitin by chitinase-catalyzed polymerization of a chitobiose oxazoline derivative was investigated by using optical microscopy in combination with TEM and SEM techniques. Platelike single crystals of α-chitin were first formed. The crystals were gradually shaped into ribbons by the rapid growth along the *a* axis with the crystalline thickness being ca. 10 nm. The α-chitin ribbons then aggregated to form bundlelike or dendritic assemblies as the ribbon concentration in solution increased. They grew into spherulites by splaying and branching, which displayed birefringence with a Maltese cross by polarization microscopic observation. The present artificial chitin spherulite, in which a number of α-chitin ribbons radiated from a common center, is completely different from the helicoidal textures composed of α-chitin microfibrils which have been known so far as a typical three-dimensional organization of chitin.

Introduction

Chitin, which is found in the exoskeleton of arthropoda for example, is one of the most abundant and widespread natural structural polysaccharides normally found in animals as cellulose in plants. Chitin attracts much attention in the fields of medicine, pharmacology, agriculture, and biotechnology because of its remarkable biological activities and utilities.[1] It is biodegradable and regarded as a promised ecomaterial, which meets the concept of "green polymer chemistry".[2]

Chitin is ideally composed of β(1,4)-linked 2-acetamido-2-deoxy-D-glucopyranose as a repeating unit. It is known, however, that the natural chitin is partially N-deacetylated in the glycan chain, and the degree of deacetylation varies depending upon the biological origin.[3] It is usually inevitable for natural chitin produced in vivo to be attacked by chitin deacetylases.[4] Therefore, in vitro synthesis should be a unique method to obtain perfect chitin that conserves the *N*-acetyl group in the every repeating unit.

A new method for the in vitro synthesis of chitin was accomplished by Kobayashi et al. in 1996.[5] In this method, a chitobiose oxazoline derivative (Chi_2NAc-oxa) was designed as an artificial substrate for Family 18 chitinase,[6] and the artificial chitin was produced by the enzymatic polymerization under weak alkaline conditions (Figure 1a). It should be noted that this method is totally different from the biosynthesis of chitin using uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) and chitin synthase (Figure 1b),[4,7] because chitinase is classified as a hydrolase enzyme. Chi_2NAc-oxa is synthesized in a good yield from *N*,*N*′-diacetylchitobiose via the three-step reaction.[5]

Self-organization of polymers into high-order structures such as lamellas, spherulites, and liquid crystals has been reported.[8,9] With respect to polysaccharides, the cholesteric liquid crystals and the helicoidal textures have been found in the tissues of living organisms.[10] Those structures were also reproduced by acid hydrolysates of cellulose and chitin.[11] The three-dimensional

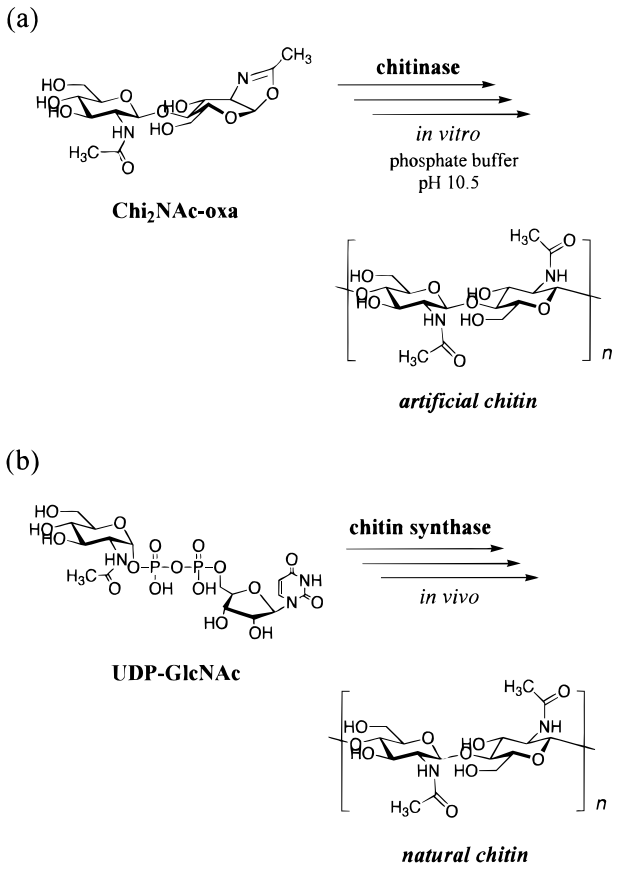

Figure 1. An abiotic method of chitin synthesis by the enzymatic polymerization of Chi_2NAc-oxa with chitinase (a) and a biotic method of UDP-GlcNAc with chitin synthase (b).

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helicoidal textures of cellulose and chitin displayed birefringence with a Maltese cross.[12] Amylose is also known to form a spherical shape of spherulite.[13]

In the present study, by taking advantage of the new synthetic method of chitin, the organization process of chitin upon in vitro enzymatic polymerization has been investigated. Thus, a new type of spherulite of chitin has been discovered as very recently disclosed for that of artificial cellulose.[14] The formation of the artificial chitin spherulites and their texture were analyzed by using phase-contrast and polarization microscopy in combination with the TEM and SEM techniques.

Experimental Section

Polymerization. A chitobiose oxazoline monomer (Chi_2-NAc-oxa) was synthesized by the method previously reported.[5] The monomer was dissolved in 10 mM phosphate buffer to adjust the concentration to be 200 mM and at pH 10.5. Enzymatic polymerization was started by adding chitinase to the solution by the amount of 1 wt % against the monomer. Commercially available chitinase (*Bacillus sp.*, Wako; EC 3.2.1.14) was used as a polymerization catalyst. The polymerization solution was kept at 30 °C.

HPLC Analysis. The monomer concentration of the polymerization solution was analyzed continuously by HPLC measurements (Shodex degasser; Tosoh pump CCPD; Shodex oven AO-50; Tosoh RI detector 8020) on a CAPCELL PAK C18 UG120 column (4.6 × 250 mm; eluent, water; flow rate, 1 mL/min; temperature, 25 °C). At each HPLC analysis, the polymerization solution was centrifuged in advance at the speed of 15 000 rpm for 2 min, and 5 μL of the supernatant solution was directly injected.

Optical Microscopy. A 20 μL aliquot of the polymerization solution was deposited onto a cavity slide and was covered by a coverslip. The temperature was regulated to be 30 °C. The microscope (Olympus IX70) was utilized at 200- or 600-fold magnification for both phase-contrast and polarization mode. At the polarization mode, the sample was observed between crossed nicols and also by using a sensitive color plate positioned between the polarizers.

Transmisson Electron Microscopy (TEM) and Electron Microdiffraction. A 10 μL aliquot of the polymerization solution was dropped on a carbon-coated grid, and an excess amount of water was sacked out with a piece of filter paper, followed by washing twice with water to remove buffer salts. After air-drying, the sample left on the grid was analyzed with a JEOL electron microscope (JEM 2000-EXII) operated at 100 kV and equipped with a GATAN camera and image intensifier (model 622-0300). The electron micrographs of unshadowed specimens were taken by diffraction contrast in the bright-field mode using low dose exposure at 10 000-fold magnification. The electron microdiffraction experiments were performed using a low dose electron probe of a 0.1−0.2 μm diameter area of the specimen. All the images and microdiffraction were recorded on Mitsubishi MEM film. The height of the single crystal was estimated by shadow-casting the sample with Pt/Pd at an angle of ca. 45° under the existence of polystyrene latex beads (diameter, 0.102 mm; Nisshin EM, Japan) as internal standard.

Scanning Electron Microscopy (SEM). The polymerization solution in 25 h was centrifuged at a speed of 2000 rpm, and the precipitate was dispersed in water with a vortex mixer. After washing twice with water, 10 μL of the resulting dispersion was deposited on a holey carbon film (microgrid for TEM preparation) and soaked up by a piece of filter paper from the backside of the grid. Then, the sample was quenched in liquid propane by a rapid freeze-drying device (KF80, Leica) and subsequently freeze-dried. Finally, the sample was coated with gold and observed by a scanning electron microscope (JEOL 6100) operated at 10−20 kV.

X-ray Diffraction. Dried solid of a water-insoluble part of the artificial chitin and authentic α-chitin from queen crab were ball-milled and pressed into a pellet (ca. 60 mg). X-ray diffraction patterns were recorded with a Rint 1400 (Rigaku) diffractometer with Cu Kα radiation generated at 40 kV and 200 mA. The scanning rate was 2.00 deg/min of the diffraction angle 2θ.

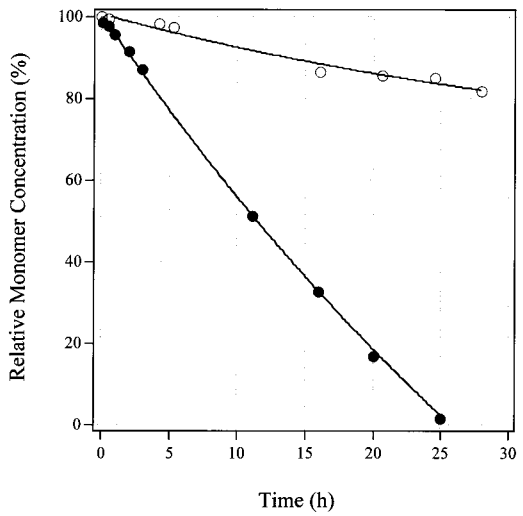

Figure 2. Time course of Chi_2NAc-oxa consumption at pH 10.5 with enzyme (●) and without enzyme (○).

Average Molecular Weight Estimation by Size-Exclusion Chromatography (SEC). The artificial chitin was converted to 6-*O*-carboxymethylchitin (CM-chitin) by the method previously reported by Tokura et al.[15] The obtained CM-chitin was analyzed by SEC (Jasco degasser DG-980-50; Jasco pump PU-980; Jasco oven CO-965; Jasco RI-930) on a COSMOSIL 5Diol-300 column (7.5 × 300 mm; eluent, 0.1 M NaCl(aq); flow rate, 1 mL/min; detector, RI; temperature, 30 °C) using pullulan ($M_w \geq 5900$, Shodex standard P-82) and chitin 6-mer (MW = 1237, Seikagaku Co.) as standard for the calibration.

Results

The enzymatic polymerization of chitin was carried out at pH 10.5 at 30 °C. Figure 2 shows the time profile of the monomer consumption. In 2 h, a number of small solids were observed in the view of optical microscopy. The solids were less than 10 μm in length and displayed little birefringence at this stage. In 3 h, the solids increased in number and size and were shaped into bundlelike and dendritic solids while displaying weak birefringence. In 10 h, they displayed the birefringence with a Maltese cross, and a distinct positive optical character was observed. That is, they were blue at the couple of the quadrants parallel to the z' axis of the sensitive color plate and orange at the other quadrants. In 25 h, when the monomer was completely consumed by the polymerization, the spherulites grew to be 20−50 μm in diameter (Figure 3).

The texture of the artificial chitin spherulites was analyzed in detail by electron microscopy. During the first 30 min, a small number of rectangular platelike solids were observed (Figure 4). Notably, they had a smooth surface and were uniformly ca. 10 nm thick. Average dimensions of the plates were 25 nm in width and 10 nm in height. The length of the plates was in the range 50 nm−1 μm. The electron microdiffraction patterns of the plates agreed well with the a^*b^* projection of the proposed orthorhombic unit cell of α-chitin having a $P2_12_12_1$ symmetry and dimensions $a = 0.474$ nm, $b = 1.886$ nm, and c (chain axis) = 1.032 nm (Figure 5).[16] The diffractogram revealed that directionalities of the length, width, and height of the plates corresponded

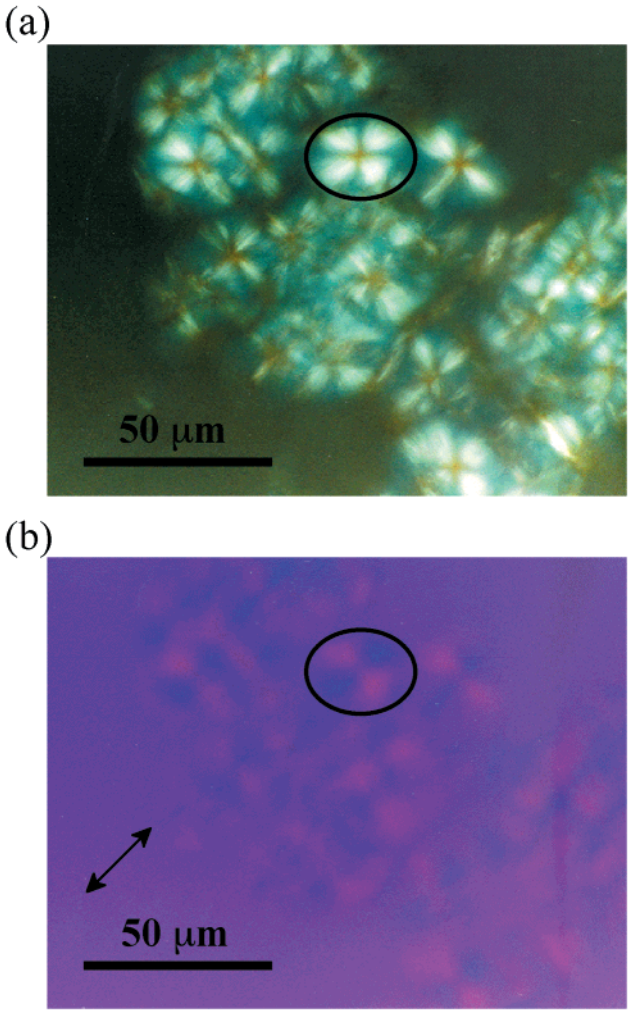

Figure 3. Polarization micrographs of artificial chitin spherulites with a positive optical character after 25 h incubation period (a) between crossed nicols and (b) between crossed nicols with a sensitive color plate. The double-headed arrow on the micrograph (b) shows the orientation of *z′* axis of the sensitive color plate.

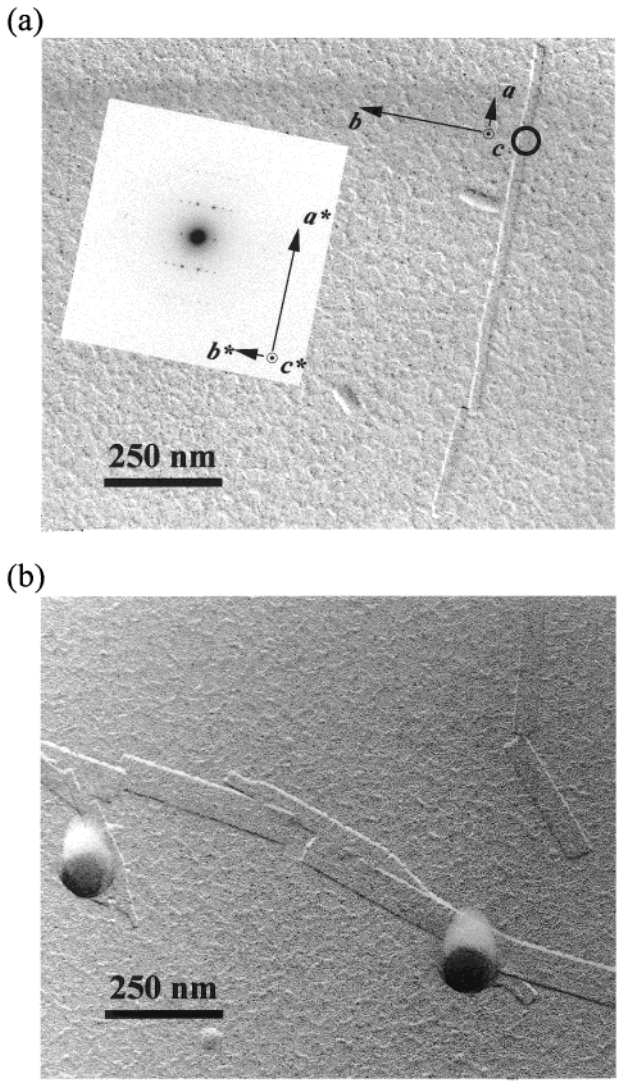

Figure 4. Electron micrographs of single crystalline ribbons of artificial chitin by TEM (a) after 30 min incubation period together with an electron microdiffraction pattern recorded in the circled area and (b) after 1 h incubation period with polystyrene latex beads as internal standard.

to those of the *a*, *b*, and *c* axis of the unit cell, respectively. α-Chitin is considered to be the thermodynamically stable form of chitin crystal with the antiparallel packing of the glycan chains with intra- and intermolecular hydrogen bonds.[17] In 1 h, a number of ribbonlike solids were observed in the view. They displayed the same electron microdiffraction patterns of α-chitin and maintained the smooth surface and the uniform thickness. The ribbonlike α-chitin showed a relatively rapid growth of the crystal along the *a* axis. Some ribbons were observed to be stacked on each other through the *ab* face, and the other adhered through the *ac* face or the *bc* face. In 3 h, bundlelike assemblies of the α-chitin ribbons were observed (Figure 6). The assemblies were approximately 10 μm large in diameter and should correspond to the bundlelike solids in the observation by optical microscopy. The texture of the artificial chitin spherulite in 25 h was observed by SEM. The spherulite was composed of the α-chitin ribbons that radiated from the center (Figure 7). The radial extinction lines were observed by polarization microscopy, which should be void spaces between the ribbons due to low packing density of the ribbons. The α-chitin ribbon was a sole product, and no other chitin crystal like microfibril was observed under the present conditions. The X-ray diffraction of the resulting product exhibited the spectrum pattern of highly crystalline α-chitin (Figure 8). These results indicate that the single crystalline ribbon of α-chitin was the majority of the whole product. It is known that α-chitin is composed of the glycan chains that are tightly bound to each other by the intermolecular hydrogen bonds involving the acetamido groups.[16] This strong hydrogen-bonding network makes it difficult for α-chitin to be dissolved in any solvent when the solubility is compared with that of β-chitin or cellulose.[18] To obtain information on the average molecular weight of the product, it was converted into a water-soluble derivative, 6-*O*-carboxymethylchitin (CM-chitin). Although some degree of degradation might occur during the reaction, it was estimated by SEC that the molecular weight of the CM-chitin distributed narrowly around 3800.

The spherulite formation was not observed during the process of the enzymatic hydrolysis of natural chitin at pH 7.0.[19] It is concluded that the enzymatic polymerization should provide very appropriate conditions for the spherulite formation.

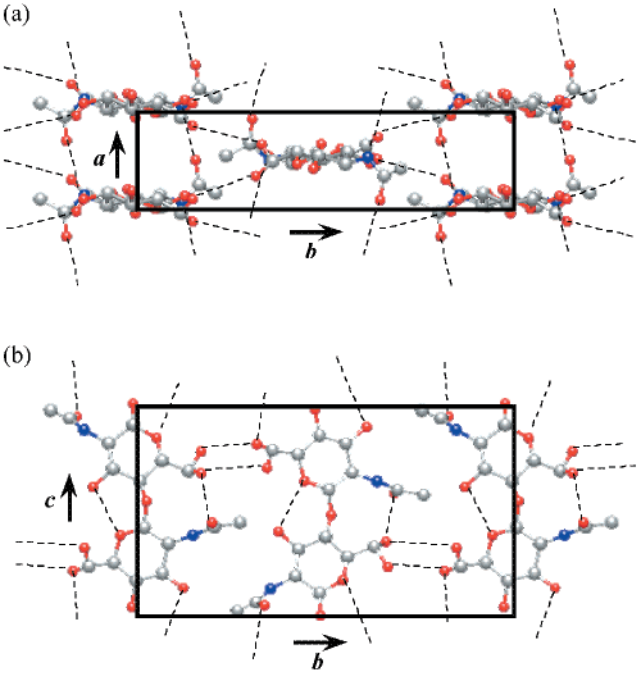

Figure 5. Unit cell structure of α-chitin proposed by Minke and Blackwell: (a) *ab* projection and (b) *bc* projection. In this proposed crystal structure, primary hydroxyl groups at the 6-position of each GlcNAc repeating unit have two possible conformations as shown in the figure, and α-chitin is considered to be a statistical mixture of them.

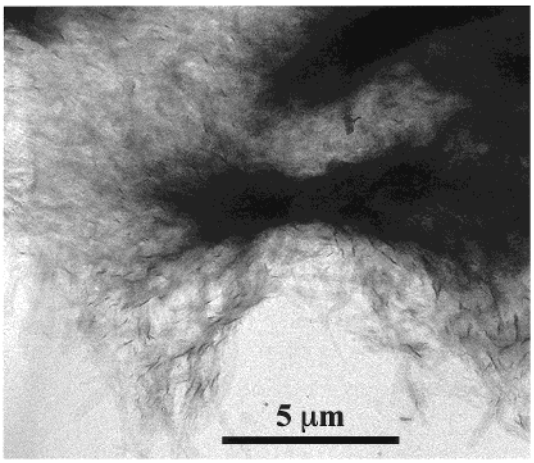

Figure 6. Electron micrograph of the bundlelike assembly of α-chitin ribbons by TEM after 3 h incubation period.

Discussion

Single Crystalline Ribbon of α-Chitin. The constant thickness (ca. 10 nm) observed on the artificial chitin ribbons provides two possibilities on the crystal structure. One is the extended chain crystal (ECC) structure that composed of chitin 20-mers, and the other is the folded chain crystal (FCC) structure[17b,c,20] by every 20 repeating moieties of the longer chains. To our knowledge, there is no previous report that confirmed the occurrence of the FCC of single crystalline α-chitin. From the result of the molecular weight estimation, it should be considered that the ECC structure is more plausible in the present case. Therefore, the artificial chitin with the monodispersed medium molecular weight was the major fraction, giving rise to a crystal form via the enzymatic polymerization. Even though the artificial chitin has some distribution in the molecular weight, the minor fractions are hardly incorporated in the single crystals, because the crystals were very stable and highly homogeneous with TEM observation.

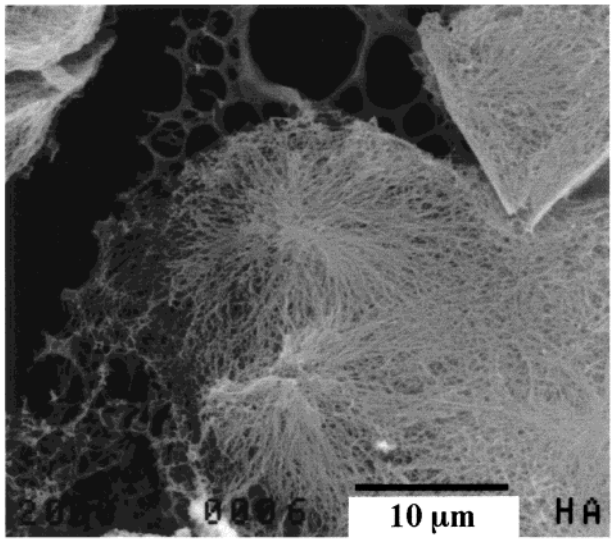

Figure 7. Electron micrograph of artificial chitin spherulite by SEM after 25 h incubation period.

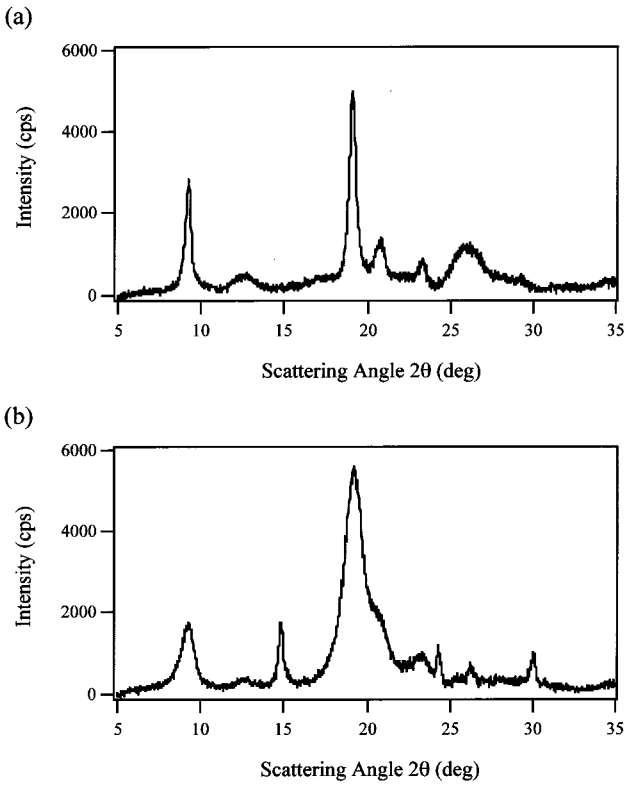

Figure 8. X-ray diffraction patterns of (a) artificial chitin and (b) natural α-chitin from queen crab.

The formation of the single crystalline ribbon of α-chitin is not unique to the artificial chitin. Helbert et al. obtained a similar ribbon of α-chitin by the recrystallization in ethanol using acid-hydrolyzed natural chitin.[21] The α-chitin ribbon was thinner and much less stable in water than the artificial chitin ribbon. The estimated thickness was less than 8 nm, which corresponds to the molecular length of chitin 16-mer. Persson et al. obtained a thicker analogue of the α-chitin ribbon using chitin 35-mer.[22] It was recrystallized in LiSCN-(aq) with poly(ethylene glycol) as precipitant at the

temperature over 200 °C. The artificial chitin ribbon, which is composed of chitin having the medium length between these two examples, exhibited high stability in water and required no severe condition for the formation. The molecular size of about 20-mer of chitin may be critical for formation of the stable single crystal in water.

Artificial Chitin Spherulite. From our observation, it was clarified that the artificial chitin spherulite is an assembly of α-chitin ribbons, and it has the texture in which a number of ribbons radiate from a common center with their *a* axis laying in the radial direction. The mechanism of the spherulite formation can be explained as follows. The concentration of the α-chitin ribbon in the polymerization solution increases upon the progress of the polymerization. The aggregation of the ribbons then occurs to form bundlelike assemblies. The assemblies are allowed to grow into spherulites by splaying and branching, which may be driven by the adhesion and stacking between the ribbons and the rapid growth along the *a* axis. This is a new type of texture discovered for the chitin spherulite. Synthetic polymers such as polyethylene (PE), poly(ethylene oxide), and *it*-polystyrene also form spherulites, which are usually obtained by a cooling process of the melted polymers.[8] No periodic circular bands of extinction were observed on the artificial chitin spherulite, indicating that the phase of torsion is not consistent between the adjacent ribbons as was observed on the synthetic polymer spherulites.[8] This difference may be due to the low packing density of the ribbons, which cannot induce the coherent growth of ribbons in their torsion. The observed coloration pattern to the four quadrants of the artificial chitin spherulite is opposite to the pattern of the PE negative spherulite, despite the similarity in texture.[8] This difference can be explained if the α-chitin ribbon possesses the largest refractive index parallel to the *a* axis. According to the molecular packing model proposed by Minke et al., the acetamido groups of α-chitin form intermolecular hydrogen bonds with alignment along the *a* axis (Figure 5).[16] It may cause the electron delocalization over a series of the hydrogen bonds, which enhances the polalizability along the *a* axis of the α-chitin ribbon to increase the refractivity. The spherulite formation of the artificial chitin has also been observed by the enzymatic polymerization using purified chitinases from *Bacillus circulans WL-12* (Figure 9).[23,24] Therefore, the organization of chitin into spherulite is induced by the enzymatic polymerization without any assistance of other proteins. There is no report on the same organization observed in the tissues of living organisms. From these viewpoints, an abiotic chitin organization into the spherulite has been realized by the abiotic methodology of the enzymatic polymerization for the first time. There were reported observations of helicoidal arrangement of microfibrils of chitin in arthropod cuticle.[10a,b] Marchessault et al. reproduced the cholesteric ordering of microfibrils in vitro.[11a,c] The microfibril was a rodlike α-chitin in contrast with the ribbonlike α-chitin of the artificial chitin. The rodlike α-chitin is likely to be composed of a bundle of longer molecular chains which were prepared by acid hydrolysis of natural chitin.[11c,25] The control of the molecular weight and its distribution is of great importance because it may determine the shape of crystals and the molecular organization into a higher-order structure.

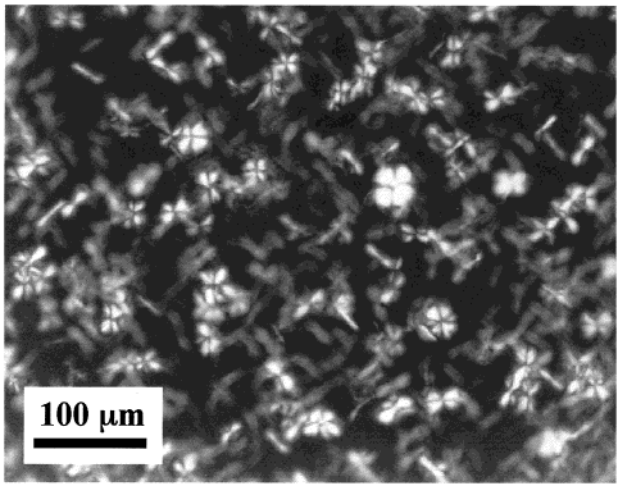

Figure 9. Polarization micrograph of the artificial chitin spherulites, which showed a positive optical character, produced by the enzymatic polymerization using a purified chitinase, chitinase C1 from *Bacillus circulans WL-12* (after 2 h incubation period).

Conclusion

It has been disclosed that the chitinase-catalyzed polymerization of Chi_2NAc-oxa is an effective method to produce ribbonlike single crystals of α-chitin and to induce organization of the ribbons into the spherulites for the first time. These results highlighted the new aspects of chitin potential and contributed to the accumulation of knowledge on the in-depth nature of chitin, which will be very useful for the design of new materials using chitin.

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