Studies on Preparation and Structural Characterization of Nano-Dispersed Chitins

(ナノ分散キチンの調製と構造解析に関する研究)

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Chapter 1
Introduction

1.1 Chitin as structural polysaccharides

Chitins are present in the outer shells of crustaceans such as crab and shrimp, the cuticles of insects and the cell walls of some fungi, coexisting with proteins and certain minerals. More than 100 million tons of chitins are biosynthesized every year.[1] Since a large amount of crab and shrimp shells are produced as food waste, further utilization of chitin as functionalized materials or commodities is highly required. The importance of chitin as a sustainable resource lies on not only its abundance but also unique structure and properties.

Isolated chitins are linear and crystalline hetero-polysaccharides consisting of N-acetylanhydroglucosamine and anhydroglucosamine units with various ratios linked by β-(1→4)-glycoside bonds as shown in Figure 1.1.

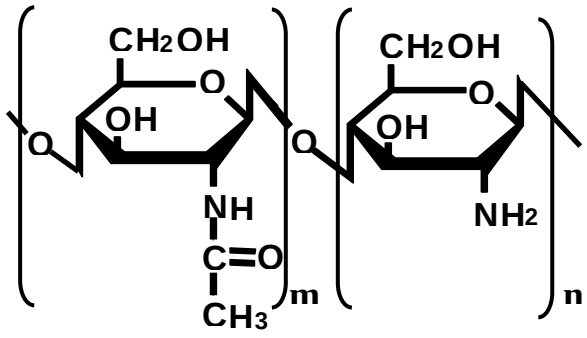

Figure 1.1. Units of chitin molecule.

As can be seen from the chitin molecules (Figure 1.1), chitin is structurally similar to cellulose, but it is a polysaccharide having acetamide or amino groups at C2-positions in place of hydroxyl groups. Chitins are the most abundant polysaccharides containing amino groups.[2] Cellulose is synthesized mainly in plants, whereas chitin is synthesized mainly in lower animals; chitins physically supporting the living bodies form hierarchical structures, in sea-animals, insects and fungi and so on, that increase in size from the simple molecules (Figure 1.1) and highly crystalline fibrils at the nano-meter level to composites at the micron level upward.

When we look into the crystal structures of chitin, three crystalline forms are known: α-, β-, and γ-chitins. In α-chitin, all molecular chains are arranged in an antiparallel mode with strong inter- and intra-molecular hydrogen bonds. On the other hand, β-chitin has a parallel chain packing mode and weak hydrogen bonds by intrasheets. γ-Chitin is less characterized than α- and β-chitins, and is reported to form two antiparallel chains and one parallel chains, i.e. a mixture (or intermediate form) of α- and β-chitins. The crystal structures of α- and β-chitins were shown in Figure 1.2.

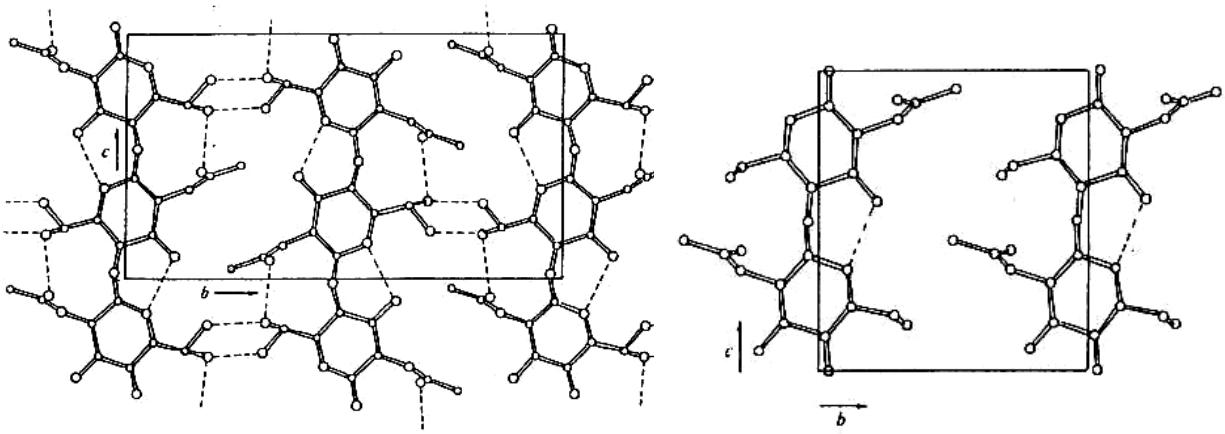

Figure 1.2. Arrangement of chitin molecules in α-chitin (left) and β-chitin (right) reported in Ref.[1] and Ref.[3].

Most natural chitins have the α-type crystal structure, while β-type chitin is present in squid pens and tubeworms.[1,4] γ-Chitin exists as the most rare crystal structure in cocoon fibers of the *Ptinus* beetle and the stomach of *Loligo*.[5] Chitins are produced from shells of crab, shrimp, tubeworm or pens of squid, which contain calcium carbonate and proteins as the two other major components. Pigments are also contained in small quantities. Furthermore, the chitin molecules are assumed to have polypeptide side chains attached covalently to some of the C2-amino groups through amide linkages. The organization of chitins in the living bodies was shown in Figure 1.3. Normally chitin could be purified from the substances by simple acid and alkali treatment for the further utilizations.

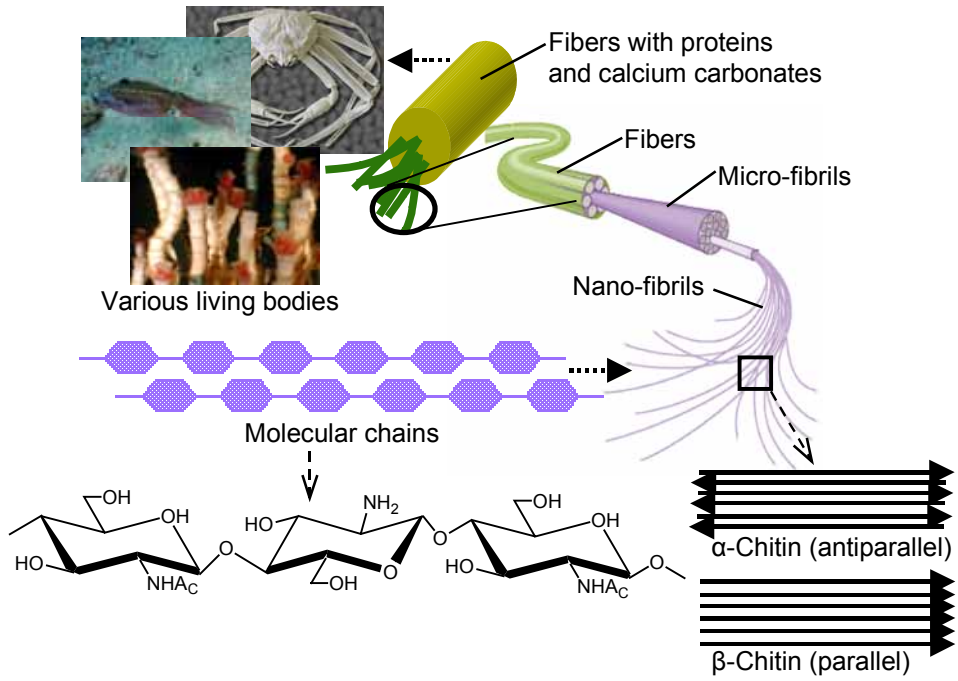

Figure 1.3. Schematic representation of the organization of chitin chains and fibrils to form hierarchical structures supporting living bodies.

1.2 Nano-dispersed chitins as highly functional materials

The presence of amino groups in chitin is highly advantageous for providing distinctive biological functions and for conducting modification reactions. A wide range of applications of chitin and chitosan (the de-acetylated product from chitin) such as photography, cosmetics, artificial skin, biomedical dressing, ophthalmology, water engineering, paper finishing, solid-state batteries, drug-delivery systems, and biotechnology etc. have been already reviewd.[6] Among these applications, combinations of the advantages of biogenesis and specific structures of chitins, e.g. nano-dispersed chitins in solvents including water to be used as highly functional materials have attracted wide attention.

New and super functionalities are expected for nano-materials and nano-material containing composites because of their superior functionalities due to their extremely large and active surface areas. On the basis of this concept, scientific and technological studies in terms of the preparation, characterization, and applications of nano-materials have been extensively studied in recent years. There are generally two routes to prepare nano-fibers of synthetic and natural polymers. The first is “bottom-up” process from polymer molecules using electrospinning, drawing, template synthesis, phase separation, sea-island composite spinning, or modified melt-blowing methods.[7-14] However, it is generally difficult to prepare highly crystalline nano-fibers by such molecular-assemble processes. The second approach is the extraction process of nano-fibers involving the downsizing of naturally occurring polymers. If nano-fibers are prepared from structural polysaccharides, such as chitin and cellulose, additional functionalities, such as biodegradability, reproducibility and biocompatibility are also introduced to the nano-fibers.

On the other hand, since a large amount of crab and shrimp shells are produced every year as food wastes, in which chitin is one of the main components, further utilization of chitin as functionalized materials or a commodity substance is highly required. The lateral dimensions of the crystalline fibrils of chitins range from 2.5 to 25 nm, depending on their biological origins.[6] Thus, due to its structural organization to form hierarchical structure supporting living bodies as shown in Figure 1.3, chitin intrinsically have the potential to be converted to crystalline nano-fibers by so-called downsizing processing. However, it is

generally quite difficult to achieve complete individualization of chitin fibrils, because these fibrils are tightly bound to each other through an endless number of hydrogen bonds in biological bodies. When extensive and high frequency (900-1000 W, 20 kHz) ultrasonication was applied to chitins for long time (30-45 min), bundles of nano-fibrillated chitin fibrils, not completely individualized ones, of 30-120 nm in width were obtained.[15]

The conventional method to prepare chitin nano-whiskers is acid hydrolysis with a hydrochloric acid, and the following repeated mechanical disintegration of the products in water:[16-21] e.g., acid hydrolysis of shrimp α-chitin with 3 M HCl at 90℃ for 1.5 h followed by high pressure homogenization of the product at 8000 psi for 10-15 cycles.[21] The obtained chitin nano-whiskers are 10-15 nm in width and 200-500 nm in length. These chitin nano-whiskers were studied as nano-composite materials for reinforcement, for nano-scaffolds in tissue engineering, and for other applications.[19, 22-27] Although the yields of the obtained chitin nano-whiskers are not described in such study, it is expected that a significant yield loss might occur by the acid hydrolysis under such harsh conditions.

Other than acid hydrolysis, the preparation of chitin nano-fibers by the bottom-up processing from chitin molecules, was carried out by electrospinning using 1,1,1,3,3,3-hexafluoro-2-propanol as the solvent[28,29] and by spraying of the chitin solutions into supercritical carbon dioxide.[30] However, as aforementioned in this part, it is generally difficult to prepare highly crystalline nano-fibers by the “bottom-up” process. Thus, new and improved method for nano-fibrillation of chitin is expected.

1.3 Promising surface modification method for nano-fibrillation of chitin

As mentioned before, chitin can be regarded as cellulose related polysaccharide with acetamide or amino groups at the C-2 position. Therefore, both primary hydroxyl groups at C-6 position and acetamide or amino groups at C-2 position, respectively, are the potential positions for surface modification reactions of crystalline fibrils.

1.3.1 Surface carboxylation by TEMPO-mediated oxidation. Catalytic oxidation using stable nitroxyl radicals such as 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) is one of the

most promising procedures for oxidation of primary alcohol groups into aldehydes and/or carboxylic acid groups.[31-34] Application of the TEMPO-mediated oxidation to polysaccharides provides highly selective conversion of primary hydroxyl groups to carboxylate ones in aqueous medium.

In our laboratory, TEMPO-mediated oxidation was applied to various celluloses. When regenerated or mercerized celluloses are used as the starting materials for the TEMPO-mediated oxidation, the aqueous cellulose slurry turns to a clear solution as the oxidation proceeds, and cellouronic acid Na salt can be obtained quantitatively.[35,36] In the case of native celluloses, however, the oxidized products were not completely soluble in water even after the oxidation with excess reagents for extended reaction times.[35-38] Later, it was found that the carboxylate and aldehyde groups formed in the TEMPO-oxidized celluloses are predominantly present at C6 position on the surfaces of cellulose I crystallites or cellulose microfibrils. Mostly individualized cellulose microfibrils dispersed in water can be obtained by TEMPO-mediated oxidation of native celluloses and successive mechanical treatment of the oxidized celluloses in water. The cellulose nano-fibers obtained by TEMPO-oxidation and successive individualization have a high density of carboxylate groups on their surface and are typically 3-4 nm in width and a few micrometers in length.[39,40] The individualized cellulose nano-fiber/water dispersions are transparent and highly viscous. The carboxylate content in the TEMPO-oxidized native celluloses is a key factor for the preparation of individualized cellulose nano-fibers.

When α- and β-chitins are oxidized by the TEMPO-NaBr system at pH 10-11 and room temperature with sufficient amounts of NaClO used as a co-oxidant, the water-insoluble chitins are converted to water-soluble products by selective oxidation of the C6 primary hydroxyl groups.[41,42] Thus, new β-(1→4)-linked polyuronic acids consisting of repeating units of the sodium salt of N-acetylglucosaminuronic acid, i.e., chitouronic acid, are obtained quantitatively. Remarkable depolymerization is inevitable during TEMPO-mediated oxidation of chitins, and the degrees of polymerization (DP) of the chitouronic acids prepared directly from α- and β-chitins are generally lower than 50.[41] When regenerated chitins and N-acetylated chitosans having degrees of N-acetylation of 0.77-0.93 are used as the starting materials in TEMPO-mediated oxidization, the chitouronic acid Na salts with DP values of ca.

300 are obtained quantitatively.[42]

Since native celluloses can be converted to highly crystalline cellulose nano-fibers by TEMPO-mediated oxidation, chitins may have the potential to be converted to chitin nano-crystals by the formation of C6 carboxylate groups selectively on the chitin crystallite surfaces. Since chitins become water-soluble chitouronic acid sodium salts by TEMPO-mediated oxidation with sufficient amounts of NaClO, the NaClO addition level should be controlled to oxidize the C6 primary hydroxyl groups present only on the chitin crystallite surfaces.

1.3.2 Surface cationization/protonation under acid conditions. Isolated and purified α- and β-chitins originally have small amounts of the C2 amino groups,[1,4] which can be cationized or protonized under acid conditions. Based on the aforementioned mechanisms to prepare cellulose nano-fibers in water by surface carboxylation of fibrils using TEMPO-mediated oxidation, in which the driving forces for the individualization of TEMPO-oxidized native cellulose might be electrostatic repulsions and/or osmotic effects between anionically-charged cellulose microfibrils, α- and β-chitins may have the potential to be converted to chitin nano-crystals by cationization/protonation of the C2 amino groups with acid followed by disintegration in water under acid conditions. Here, a sufficient number of cationic C2 ammonium groups must be present on the chitin crystallite surface. If this hypothesis is applicable, the opposite charges to TEMPO-oxidized cellulose nano-fibrils, cationic charges, will be the candidate in this case. Hence, nano-dispersed chitins will have some advantageous points of “no chemical reactions” which is significant for safety issue in applications to animals and human beings, and suitable for highly functionalized applications in life science and bio-fields.

The promising surface modification routs to provide surface charges of chitin either by carboxylation or cationization will be discribed in Figure 1.4.

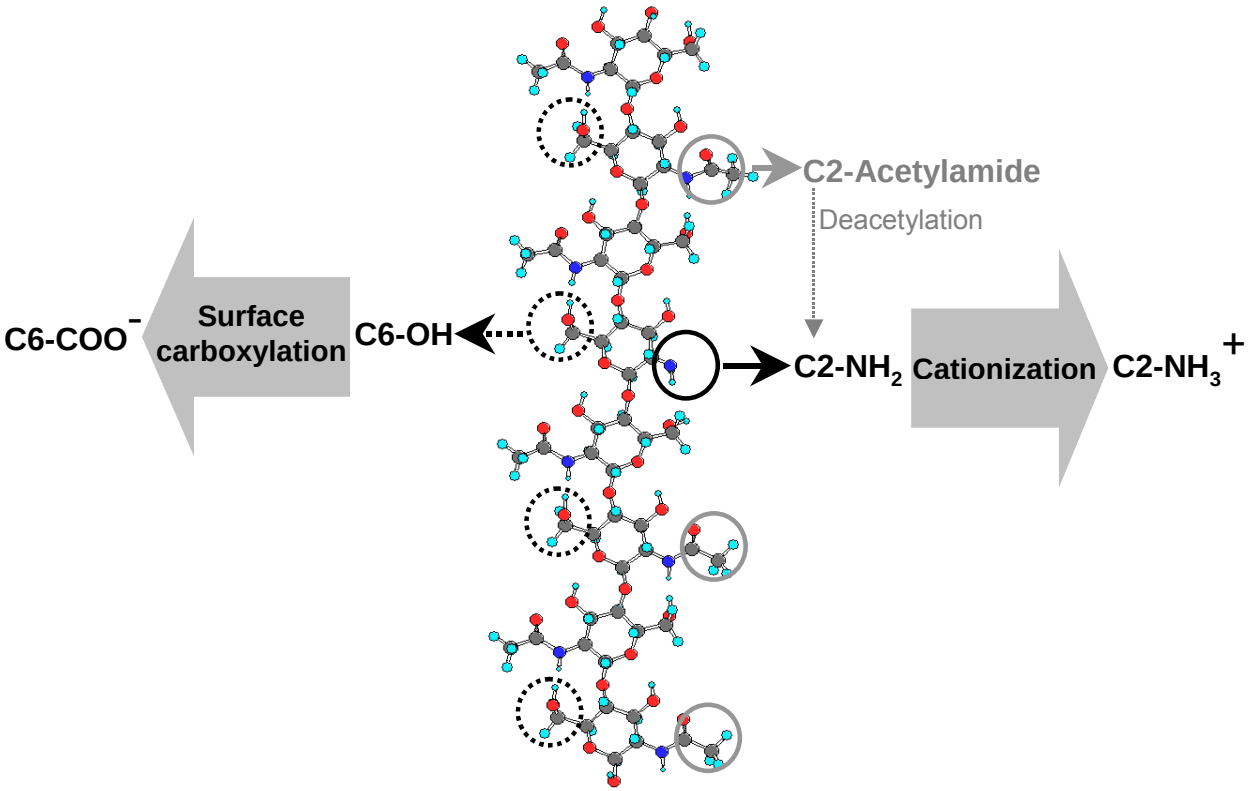

Figure 1.4. Sketch map of possible routs for surface modification of chitin fibrils. Surface carboxylation may be achieved by TEMPO-mediated oxidation, while cationization may be possible by simple disintegration in water under acidic conditions.

1.4 Objective of this study

Nano-dispersed chitins have remarkable advantages in terms of biodegradability, biocompatibility and bioactivity, together with extreme large and active surfaces due to the nano-size effects. Thus, nano-dispersed chitins have great potential to be used as highly functional materials in life science and bio-field. The conventional methods to prepare chitin nanocrystals, either by bottom-up processing such as electro-spinning, or downsizing with acid hydrolysis, were always accompanied by decreases in crystallinity and yields. Thus, in

this study, new methods were explored to prepare nano-dispersed chitins by mild and environmental-friendly reaction systems. To obtain nano-dispersed chitins with high yields under moderate conditions maintaining the original high crystallinity, and the characterization of nano-dispersed chitins was also objected.

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Chapter 2
Preparation of Chitin Nano-Whiskers from α-Chitins by TEMPO-Mediated Oxidation

2.1 Introduction

In this chapter, TEMPO-mediated oxidation was applied to crab shell α-chitin. Relationships between the amount of NaClO added in the TEMPO-mediated oxidation and either the weight ratio of the water-insoluble fraction in the TEMPO-oxidized chitin, its carboxylate content, crystallinity and crystal size, or its swelling behavior in water, were first clarified. Second, the TEMPO-oxidized chitins were subjected to ultrasonic treatment in water to prepare chitin nano-whiskers. The shapes, lengths, and widths of the chitin nano-whiskers dispersed in water were evaluated by transmission electron microscopy.

2.2 Materials and Methods

2.2.1 Materials. A commercial α-chitin powder (passed through 4 mesh sieve) originating from crab shells (Wako Pure Chemicals, Co., Japan) with a degree of N-acetylation of 0.93, determined by elemental analysis, was used as the starting material. TEMPO, sodium bromide, a 2 M sodium hypochlorite solution, and other chemicals and solvents were of laboratory grade (Wako) and used without further purification.

2.2.2 TEMPO-mediated oxidation. Chitin (1 g) was suspended in water (100 mL) containing TEMPO (0.016 g, 0.1 mmol) and sodium bromide (0.1 g, 1 mmol). The oxidation of chitin was started by adding a desired amount of NaClO solution (0-10 mmol of NaClO per gram of chitin). The pH of the slurry was maintained to be 10 at room temperature by continuous addition of 0.5 M NaOH using a pH-Stat titration system. When no consumption

of the alkali was observed, the oxidation was quenched by adding a small amount of ethanol to the mixture. After the pH was adjusted to 7 with 0.5 M HCl, the mixture was centrifuged at 12,000 g for 5 min, and the supernatant was removed by decantation. The water-insoluble fraction thus obtained was washed thoroughly with water by repeated centrifugation and the successive decantation of the supernatant. A part of the water-insoluble fraction was freeze-dried for analyses, and the rest was stored at 4°C as a wet TEMPO-oxidized chitin sample before use.

2.2.3 Determination of carboxylate and aldehyde contents. The carboxylate content of the TEMPO-oxidized chitins was determined by the electrical conductivity titration method.[1] To a dried sample (0.2 g) were added water (60 mL) and a small amount of 0.5 M NaOH to adjust the pH to 9. The mixture was stirred for 30 min to prepare a well-dispersed slurry. Then, 0.1 M HCl was added to the mixture to set the pH in the range of 2.5-3.0. A 0.05 M NaOH solution was added at a rate of 0.1 mL/min up to pH 11 by using a pH-Stat titration system. The conductivity and pH curves thus obtained reflected the content of both carboxylate and C2 amino groups in the TEMPO-oxidized chitins, because the carboxylate groups were apparently indistinguishable from the amino groups in the titration curves. Hence, the carboxylate content was obtained by subtracting the content of amino groups from the measured titration values for the TEMPO-oxidized chitins, which were confirmed by elemental analysis and infrared spectroscopy, based on the assumption that no N-deacetylation occurs during the TEMPO-mediated oxidation as described later. The aldehyde content was measured according to the following standard method.[1,2] The TEMPO-oxidized chitins were further oxidized with sodium chlorite at pH 4-5 for selective conversion of the aldehyde groups in the samples to carboxyl groups, and the carboxyl content was determined by the above-mentioned electrical conductivity titration method. To a freeze-dried TEMPO-oxidized chitin (0.5 g), were added water (40 mL), $NaClO_2$ (0.9 g) and 5 M CH_3COOH (5 mL). Oxidation was carried out by stirring the mixture at room temperature for 48 h, followed by washing thoroughly with water by centrifugation. The carboxylate groups formed by the $NaClO_2$ oxidation were regarded as aldehyde groups present in the original TEMPO-oxidized chitins.

2.2.4 X-ray diffraction analysis. The original and TEMPO-oxidized chitins were converted to pellets using a disk apparatus for IR measurement and subjected to X-ray diffraction analysis from 5° to 35° diffraction angle 2θ using the reflection method by means of a Rigaku RINT 2000 with Ni-filtered Cu Kα radiation (λ 0.15418 nm) at 40 kV and 40 mA. Since all of the TEMPO-oxidized chitins prepared in this chapter had X-ray diffraction patterns of the α-chitin type, the crystallinity was calculated from the peak intensity I_{110} and the baseline intensity $I_{amorphous}$ at the diffraction angles of 19.6° and 16.0°, respectively.[3] The crystal sizes of the (020) and (110) planes[4] were measured from the widths at half-heights of the diffractions peaks at 9.6° and 19.6°, respectively, using Scherrer's equation.[5]

2.2.5 Water-retention value. The wet TEMPO-oxidized chitin was centrifuged on a glass filter with ca. 100 μm pore size at 3000 g and 20°C for 30 min. The water-retention value (WRV) was calculated using the following equation:

$$WRV\ (\%) = 100 \times (Ww - W_d) / W_d$$

where Ww is the mass of the wet sample after centrifugation and W_d is that after drying of the wet sample at 105°C for 3 h.

2.2.6 Sonication of TEMPO-oxidized chitin/water slurries. The wet TEMPO-oxidized chitin was suspended in water at 0.1% consistency. Ultrasonic treatment was applied to the slurry (10 mL) for 1 min using an ultrasonic generator (US-300T, Nihonseiki Co., Japan).

2.2.7 FT-IR spectroscopy. Thin films were prepared by casting the original chitin/water and TEMPO-oxidized chitin/water dispersions, which were prepared from the slurries by ultrasonic treatment, on a Teflon plate followed by drying overnight at 50°C. The sodium carboxylate groups in the TEMPO-oxidized chitin films were converted to free carboxyl groups by immersion of the films in 0.01 M HCl for 1-2 h followed by washing thoroughly with water. The air-dried films were further vacuum-dried at 50°C for 24 h. FT-IR spectra of the films of the original and TEMPO-oxidized chitins with free carboxyl groups were recorded on a Nicolet Magna 860 (Madison, WI) in transmission mode.

2.2.8 Microscopic observations. Slurries or dispersions of TEMPO-oxidized chitins in water were observed using an optical microscope (Olympus BX50) equipped with a phase-contrast lens (Olympus UPlanFLN-PH), cross-polarizers in some cases, and a digital camera (Olympus PD20). A 10 μL aliquot of the 0.02% (w/v) TEMPO-oxidized chitin/water dispersion was mounted on a glow-discharged carbon coated electron microscopy grid. The excess liquid was absorbed by filter paper, and 1 drop of 2% uranyl acetate negative stain was added before drying. The excess solution was blotted with a filter paper and allowed to dry naturally by evaporation. The sample grid was observed at 100 kV using a JEOL electron microscope (JEM 2000-EXII). All micrographs were taken on Fuji FG film.

2.3 Results and Discussion

2.3.1 Oxidation of chitin. The C6 primary hydroxyl groups of chitin are selectively oxidized to carboxylate groups via the aldehyde structure by TEMPO-mediated oxidation. Chitin can be converted to the corresponding water-soluble polyuronic acid with partial depolymerization, when a sufficient amount of NaClO is added to the chitin/water slurries at pH 10-11 in the oxidation.[6,7] The outline of the reaction is shown in Figure 2.1. Since general α-chitins originating from crab and shrimp shells have high crystallinity like native celluloses,[4] it might be possible to convert to chitin nano-whiskers by formation of the C6 carboxylate groups selectively on the surfaces of the chitin crystallites by TEMPO-mediated oxidation. The amount of NaClO added to the chitin/water slurries might be one of the key factors controlling the amount and location of carboxylate groups formed in chitin.

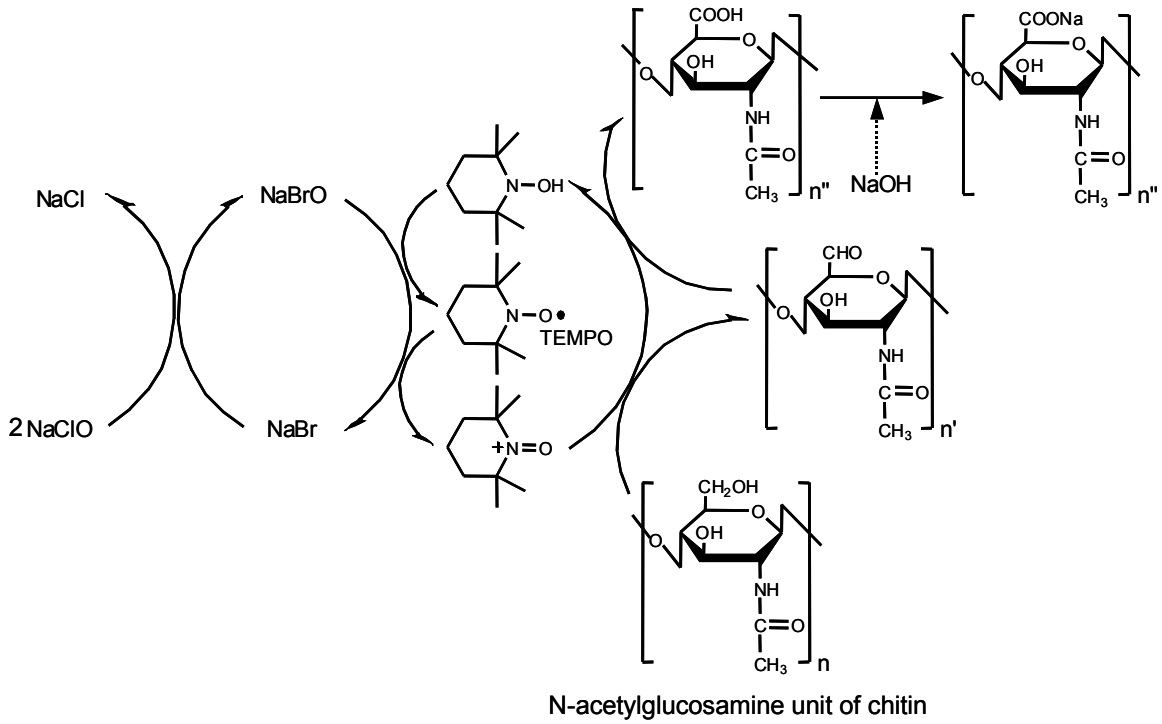

Figure 2.1. TEMPO-mediated oxidation of the N-acetylglucosamine units of chitin.

Figure 2.2 shows the relationships between the amount of NaClO added as the co-oxidant and either reaction time or weight percentage of the water-insoluble fraction in the TEMPO-oxidized chitin. The weight percentages were expressed as the weight ratios of the water-insoluble fractions obtained with respect to the original chitin used as the starting material. As the amount of NaClO added was increased, the time required for oxidation increased as well, and inversely the weight percentage of the water-insoluble fraction decreased. When the amount of NaClO added was 5.0 mmol/g of chitin, the oxidation time and the water-insoluble fraction were about 90 min and 90%, respectively. At 7.5 and 10 mmol NaClO per gram of

chitin, more than 50% of the original chitin was converted to the water-soluble fraction. The chitin powder in the slurry was completely dissolved in the aqueous oxidation medium at 20 mmol NaClO per gram of chitin.[6,7] The ^{13}C NMR spectrum of this product showed that the chitin was completely converted to the sodium salt of chitouronic acid mostly consisting of the same repeating unit, as illustrated in Figure 2.1. The relationship between the amount of NaClO added and the weight percentage of the water-insoluble fraction is different from those of native celluloses. The complete dissolution of native celluloses in the aqueous reaction media cannot be achieved by TEMPO-mediated oxidation, even after the addition of a large amount of NaClO or extended reaction time.[1,8]

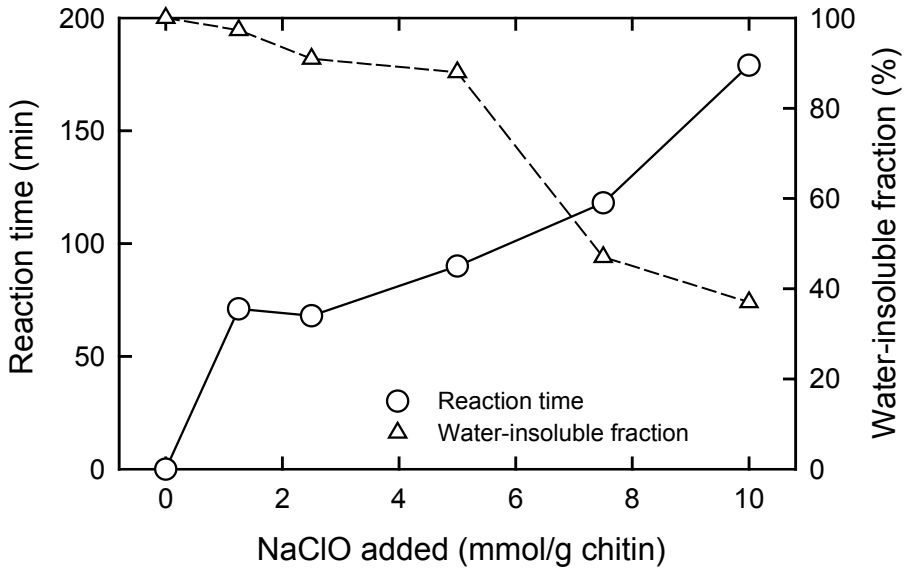

Figure 2.2. Relationships between the amount of NaClO added in the TEMPO-mediated oxidation of chitin and either the total reaction time or the weight ratio of the water-insoluble fraction.

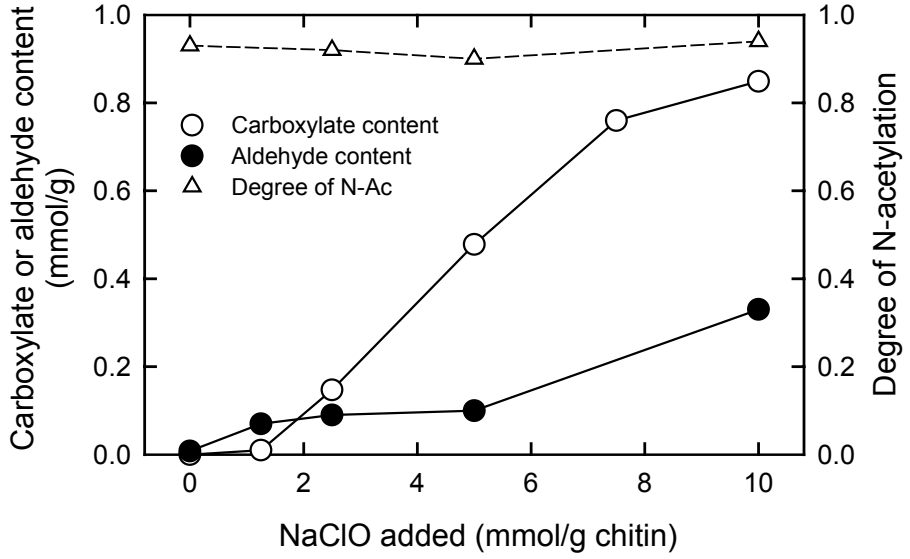

Figure 2.3. Relationships between the amount of NaClO added in the TEMPO-mediated oxidation of α-chitin and either the carboxylate or aldehyde content of the water-insoluble fraction. The degree of N-acetylation is also plotted.

The carboxylate and aldehyde contents of the water-insoluble fractions in the TEMPO-oxidized chitins are plotted with respect to the amount of NaClO added (Figure 2.3). The carboxylate content increased almost linearly, while certain amounts of aldehyde groups were also present in the water-insoluble fractions. The total contents of carboxylate and aldehyde groups were 0.58 and 1.18 mmol/g at 5.0 and 10 mmol of NaClO per gram of chitin, respectively, which roughly correspond to 12% and 24% of the C6 primary hydroxyl groups of the original chitin to be oxidized to either carboxylate or aldehyde groups by the oxidation.

From the reaction mechanism in Figure 2.1, 2 mol of NaClO is required to oxidize 1 mol of the C6 primary hydroxyl group to a carboxylate group. However, the carboxylate and

aldehyde groups formed in the water-insoluble fractions were lower than those stoichiometrically anticipated. Only about 20% of NaClO added at both 5.0 and 10 mmol of NaClO additions per gram of chitin were consumed to form carboxylate and aldehyde groups in the water-insoluble fractions. Moreover, only 20% of the NaOH added during these NaClO additions was consumed to neutralize the carboxylate groups formed in the water-insoluble fractions. These results show that significant amounts of NaClO and NaOH were consumed by side reactions other than the oxidation of the C6 primary hydroxyl groups and neutralization of the carboxyl groups formed in the water-insoluble fractions, respectively, e.g., formation of the water-soluble fractions and their depolymerization during TEMPO-mediated oxidation of chitin. Since the oxidation was carried out in an open system, a part of the NaOH added might have been consumed for the neutralization of atmospheric CO_2 dissolved in the slightly alkaline reaction medium.

The degrees of N-acetylation of the water-insoluble fractions were mostly unchanged with respect to the amount of NaClO added (Figure 2.3), showing that nearly no N-deacetylation occurs on the chitin molecules during the oxidation, irrespective of the amount of NaClO added. Hereafter, the water-insoluble fractions obtained from the TEMPO-oxidized chitins by removal of the water-soluble fractions using centrifugation and washing with water are simply referred to as that “TEMPO-oxidized chitins” for convenience.

The FT-IR spectra of the TEMPO-oxidized chitins are shown in Figure 2.4. The sodium carboxylate groups in the products were converted beforehand to free carboxyl groups, which have an absorption band around 1740 cm^{-1}. The absorption ratios A1740/A1030 using the baseline proposed by Shigemasa et al.[9] corresponded well to the carboxylate contents of the TEMPO-oxidized chitins in Figure 2.3, where the band at 1030 cm^{-1} is due to the C-O stretching vibration of the chitin skeleton and used as an internal standard.[10] The OH stretching bands at 3480 and 3450 cm^{-1} and the C-O stretching band at 1070 cm^{-1} of the original chitin seem to be saturated, resulting in lower relative absorptions than those of the TEMPO-oxidized chitins. The degrees of N-acetylation can also be evaluated from the FT-IR spectra using the absorption ratio A1560/A1030 and base lines in Figure 2.4, where the absorption at 1560 cm^{-1} is due to the amide II band.[8] The ratios of A1560/A1030 for the original and TEMPO-oxidized chitins were 0.71-0.75. These values corresponded to the

degrees of N-acetylation of 0.90-0.95, when the reported correlation equation was used.[10] Thus, not only elemental analysis but also FT-IR analysis shows that the TEMPO-oxidized chitins can be regarded as having the same degrees of N-acetylation or the same amino group content for each repeating unit as that of the original chitin.

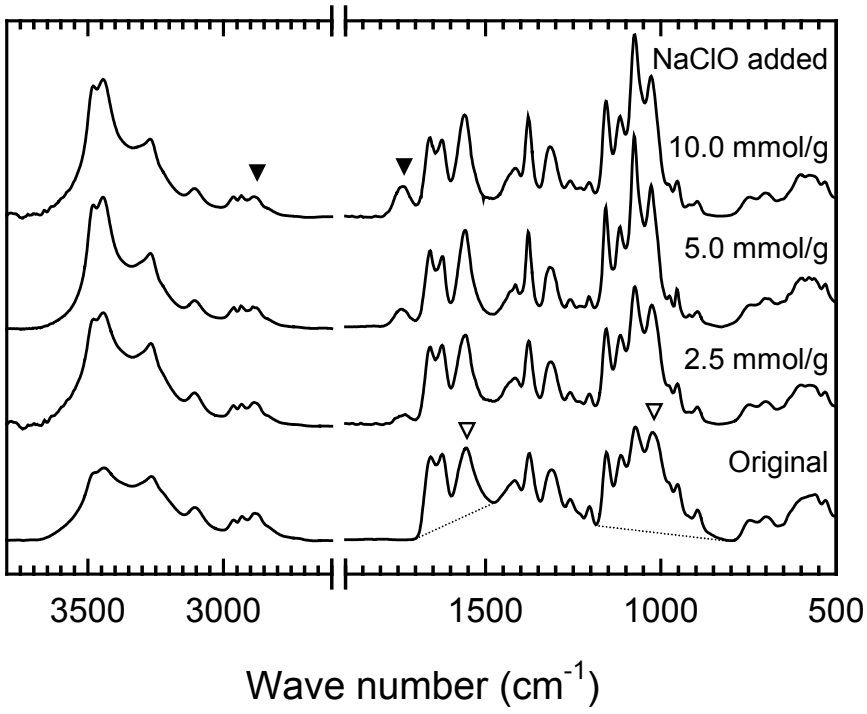

Figure 2.4. IR spectra of the water-insoluble fractions in the TEMPO-oxidized α-chitins prepared with various amounts of NaClO.

The C-H stretching band at 2880 cm^{-1} slightly but undoubtedly decreased with TEMPO-mediated oxidation. Since the C6 primary hydroxyls are converted to C6 sodium carboxylate groups by oxidation, the band at 2880 cm^{-1} may be due to the C-H stretching vibration at the C6 primary hydroxyls.

2.3.2 Changes in crystallinity and crystal size. Figure 2.5 shows the X-ray diffraction patterns of the original and TEMPO-oxidized chitins. All the TEMPO-oxidized chitins, as well as the original chitin, had diffraction peaks at 9.6°, 19.6°, 21.1°, and 23.7°, which are typical for the α-chitin structure.[4] Hence, the original α-chitin structure was maintained in the TEMPO-oxidized chitins, although the peak intensity due to the (020) plane clearly decreased as a result of oxidation. The crystal sizes of the (020) and (110) planes are plotted in Figure 2.6 together with the crystallinity of the original and TEMPO-oxidized chitins.

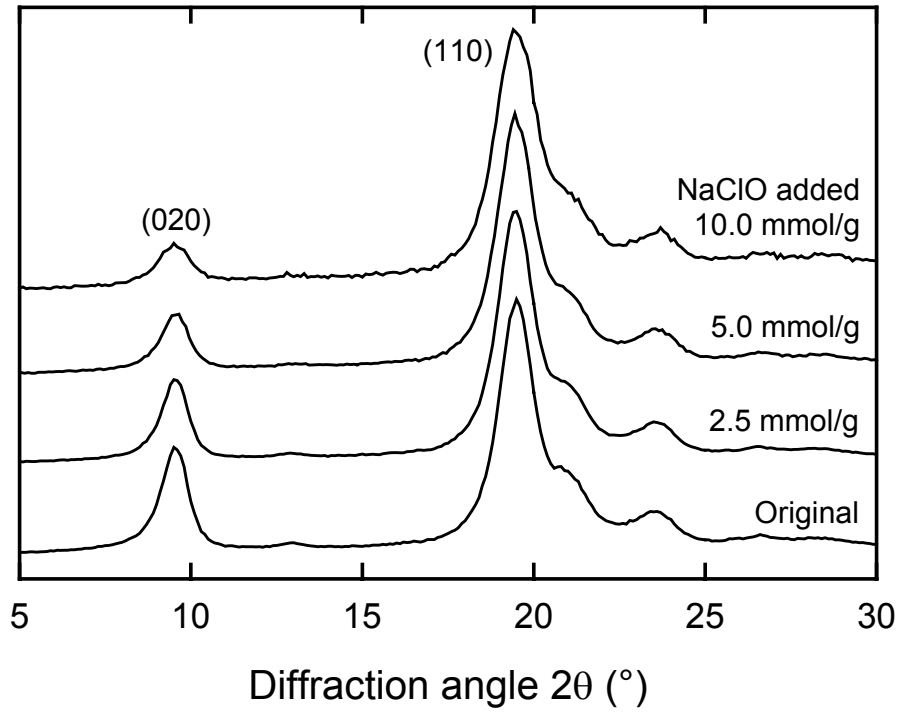

Figure 2.5. X-ray diffraction patterns of the TEMPO-oxidized α-chitins prepared with various amounts of NaClO.

As shown in Figure 2.6, the crystal sizes of both the (020) and (110) planes decreased from 8.6 and 7.0 nm, respectively, in accordance with the increased amount of NaClO added during the oxidation. These crystal sizes of the α-chitin structure in the TEMPO-oxidized chitins indicate that chitin nano-whiskers 6-9 nm in width may be obtained, if they can be individualized by the TEMPO-mediated oxidation and the successive mechanical treatment in water. The crystallinity was maintained to be approximately 93%, irrespective of the amount of NaClO added, when the baseline height at 16.0° and the diffraction peak at 19.6° due to (110) plane were used as the amorphous and crystal components, respectively.[3]

Although the crystallinity was mostly unchanged, the peak intensity at 9.6° due to (020) plane was clearly decreased as a result of the oxidation. The (020) plane may therefore be preferentially oriented parallel to the surface in the disk pellet, and this plane orientation may decrease with oxidation. The slight decreases in the crystal sizes in Figure 2.6 show that the chitin molecules on the crystallite surfaces are partially transferred to the water-soluble fractions by TEMPO-mediated oxidation, whereas, the high crystallinity of the original chitin is maintained. The crystallinity and crystal size data substantiate the fact that the C6 carboxylate groups formed in the water-insoluble fractions of the TEMPO-oxidized chitins are present only on the crystallite surfaces.

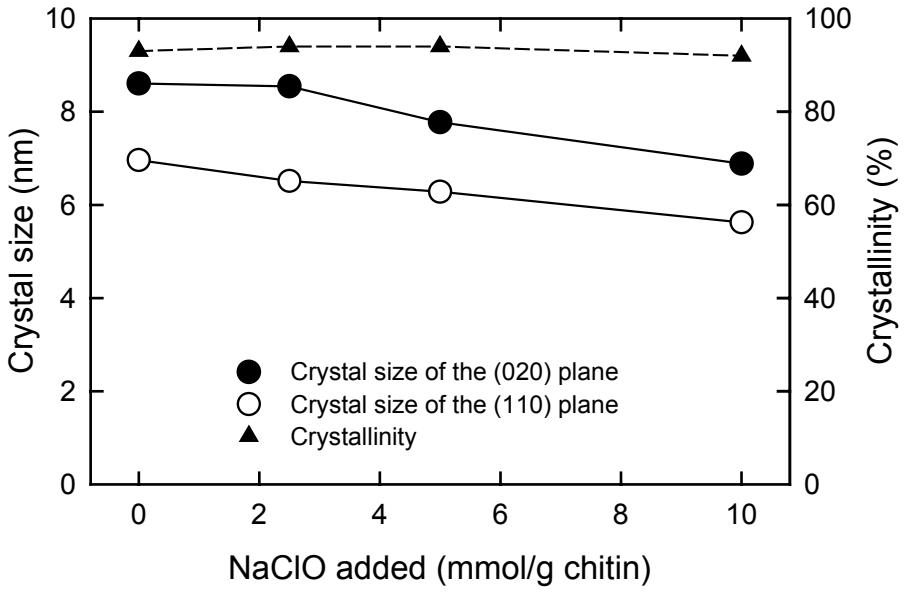

Figure 2.6. Changes in crystallinity and crystal size of the TEMPO-oxidized α-chitins prepared with various amounts of NaClO.

2.3.3 Swelling behavior of TEMPO-oxidized chitins in water. The TEMPO-oxidized chitins prepared with various amounts of NaClO were subjected to measurement of water-retention values for evaluation of their swelling behavior in water (Figure 2.7). The carboxylate contents in Figure 2.3 are also depicted in this figure. The water-retention values corresponded well to the carboxylate contents of the TEMPO-oxidized chitins, showing that their swelling behavior in water is strongly governed by the amount of hydrophilic sodium carboxylate groups formed. The TEMPO-oxidized chitins prepared with 7.5 and 10 mmol of NaClO per gram of chitin were strongly swollen in water, like gels, and had water-retention values of 1340% and 1880%, respectively.

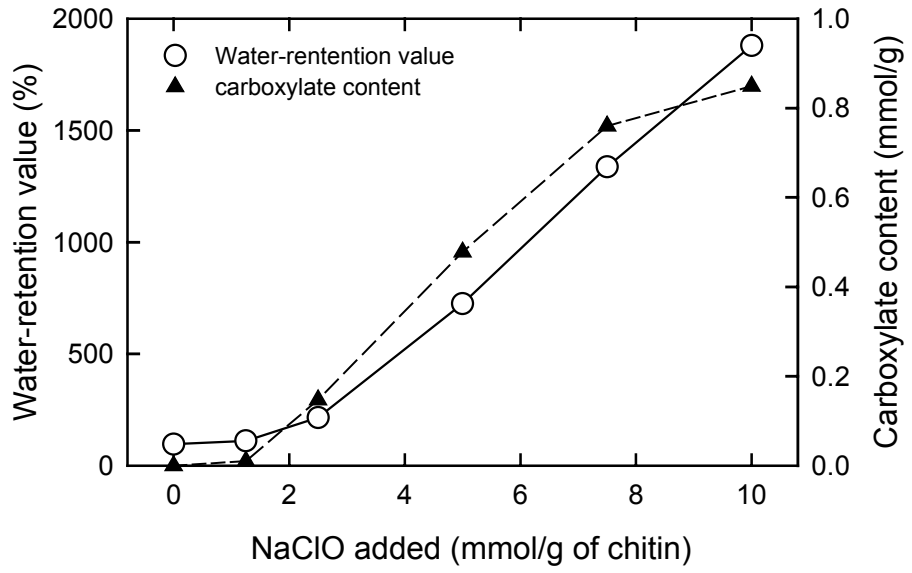

Figure 2.7. Water-retention value and carboxylate content of the TEMPO-oxidized α-chitins prepared with various amounts of NaClO.

The optical micrographs of the TEMPO-oxidized chitins in water are shown in Figure 2.8. The flake form of the original chitin was maintained after the oxidation with 2.5 mmol of NaClO per gram of chitin. When the amount of NaClO added was increased to 5.0 mmol/g of chitin, many cracks were formed in the oxidized chitin flakes and some fibrils were detected around the flakes. A strongly swollen structure was observed for the TEMPO-oxidized chitin prepared with 10 mmol of NaClO per gram of chitin. These micrographs were well consistent with the water retention values shown in Figure 2.7.

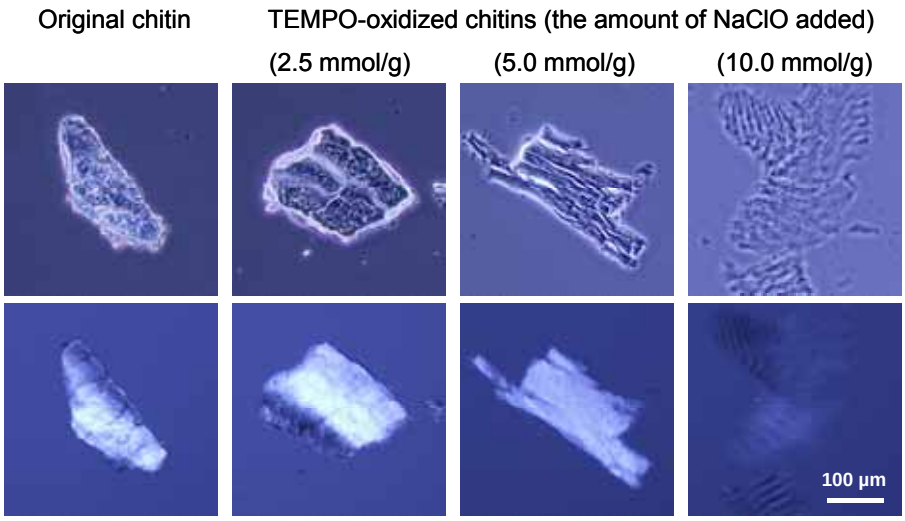

Figure 2.8. Optical micrographs of the TEMPO-oxidized α-chitins prepared with various amounts of NaClO (before ultrasonic treatment). Taken with (lower) or without (upper) cross polarizers.

2.3.4 Preparation of chitin nano-whiskers dispersed in water. The TEMPO-oxidized chitins as well as the original chitin were subjected to ultrasonic treatment. Photos of the slurries or dispersions in water are shown in Figure 2.9. The original chitin flakes sedimented spontaneously at the bottom of the bottle without any dispersion or swelling in water, even after extended ultrasonic treatment. When 2.5 mmol of NaClO per gram of chitin was used, the TEMPO-oxidized chitin dispersed well in water, although the dispersion had a high turbidity; significant amounts of bundles or aggregates of chitin crystallites are still present in the dispersion.

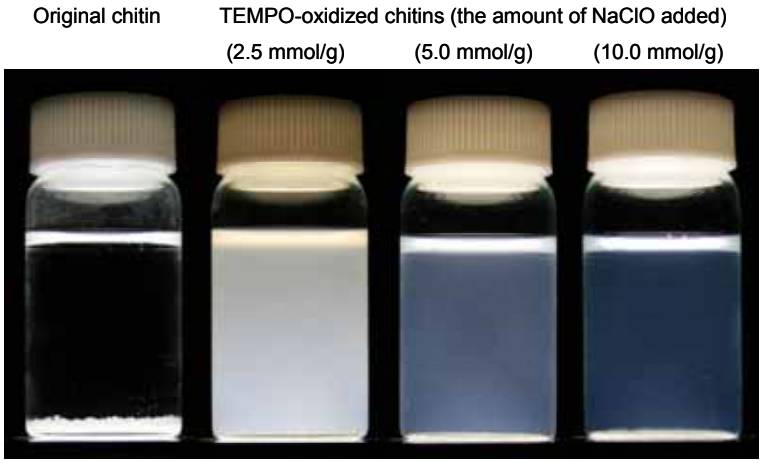

Figure 2.9. Photographs of the TEMPO-oxidized α-chitins after disintegration in water for 1 min using an ultrasonic homogenizer.

When the TEMPO-oxidized chitins were prepared with 5.0 and 10 mmol of NaClO per gram of chitin, the oxidized products were dispersed well in water by ultrasonic treatment, providing translucent dispersions. The extension of the ultrasonic treatment time for these dispersions had no influence on the dispersion behavior, and the turbidities were nearly unchanged. These dispersions showed flow birefringence between polarized films, indicating the presence of nano-whiskers having optical anisotropy. Thus, the carboxylate content in the TEMPO-oxidized chitins is a significant factor affecting the transparency of the dispersions. The formation of anionic C6 carboxylate groups on the chitin crystallite surfaces by TEMPO-mediated oxidation may enhance the individualization of the crystallites by simple mechanical treatment, such as sonication through electrical repulsion between the crystallites

and/or the osmotic effect in a similar manner to that of cellulose nano-fibers prepared by TEMPO-mediated oxidation.

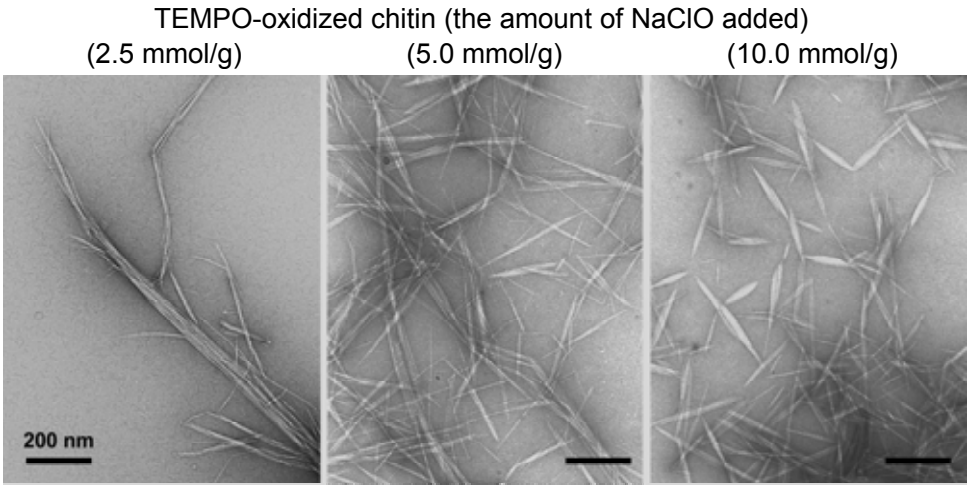

Figure 2.10. Transmission electron micrographs of TEMPO-oxidized α-chitin nano-whiskers prepared under different conditions.

Transmission electron microscopy (TEM) images of the dispersions thus obtained are shown in Figure 2.10. When the amount of NaClO added was 2.5 mmol/g of chitin, large bundles consisting of needle-like chitin crystallites with nano-scale widths were frequently observed, and some branches of smaller bundles or partly individualized fibers were attached to the large bundles. The number of individualized chitin nano-whiskers increased for the TEMPO-oxidized chitin prepared with 5.0 mmol of NaClO per gram of chitin, although bundles of the fibers still existed to some extent in the dispersion. The individualization of the fibers further proceeded for the TEMPO-oxidized chitin prepared with 10 mmol of NaClO per gram of chitin. However, the lengths of these fibers clearly became shorter. Some of these

whiskers had spindle shapes, indicating that chitin molecules were partly transferred from the edge surfaces of the whiskers to the water-soluble fractions during TEMPO-mediated oxidation under such relatively harsh conditions. This is the reason for the low weight ratio (37%) of the water-insoluble fraction for this TEMPO-oxidized chitin (Figure 2.2). These TEM images correspond well to the turbidities of the dispersions in Figure 2.9; the more the individualized fiber/whisker fraction increases, the more transparent the dispersion becomes.

The distribution of fiber lengths of the TEMPO-oxidized chitins prepared with 5.0 and 10 mmol of NaClO per gram of chitin is shown in the upper graph in Figure 2.11, where the number of bundles was precluded from the calculation. The fiber lengths ranged from 50 to 500 nm for the TEMPO-oxidized chitins prepared with both 5.0 and 10 mmol of NaClO per gram of chitin. The weight average lengths were 340 and 270 nm for the TEMPO-oxidized chitins prepared with 5.0 and 10 mmol of NaClO per gram of chitin, respectively. Thus, the more NaClO added in the TEMPO-mediated oxidation of chitin, the shorter the chitin nanowhisker length.

The distribution of fiber widths for the TEMPO-oxidized chitins prepared with 2.5, 5.0, and 10 mmol of NaClO per gram of chitin is depicted in the lower graph in Figure 2.11, where again the number of bundles was precluded from the calculation. The TEMPO-oxidized chitin nano-whiskers prepared with 2.5 and 5.0 mmol of NaClO had fiber widths smaller than 15 nm, and the average widths were 10 and 8 nm, respectively. These values were consistent with those obtained by X-ray diffraction, as shown in Figure 2.6. On the other hand, the TEMPO-oxidized chitin prepared with 10 mmol of NaClO had fiber widths ranging from 7 to 20 nm, and the average was 13 nm. It is difficult to distinguish individualized chitin nano-whiskers from bundles by TEM. Inter-whisker linkages may have formed to some extent by electrostatic interactions between anionic C6 carboxylate groups and cationic C2 ammonium groups on the surfaces of chitin whiskers, resulting in the larger width of the whiskers. However, details are unknown at this moment.

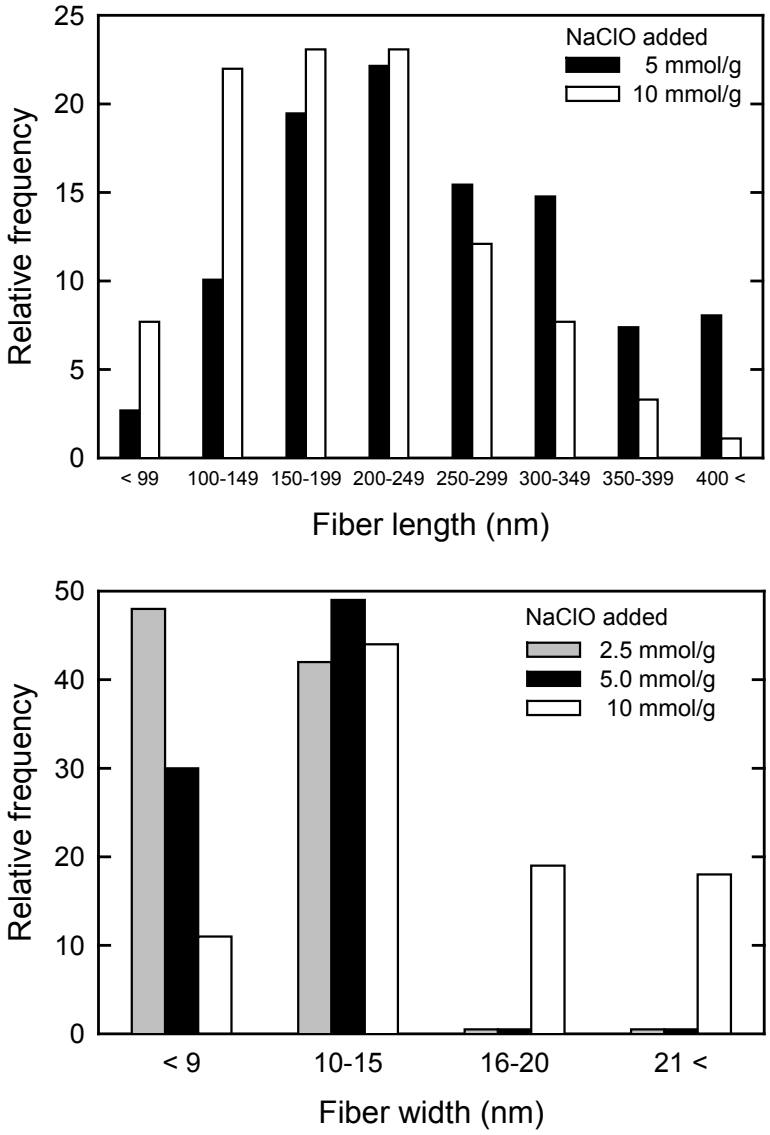

Figure 2.11. Length and width distribution of chitin nano-whiskers prepared from α-chitin by TEMPO-mediated oxidation.

In conclusion, highly crystalline chitin nano-whiskers dispersed in water are prepared by TEMPO-mediated oxidation of α-chitin and the following ultrasonic treatment. The carboxylate content of the TEMPO-oxidized chitins or the amount of NaClO added in the oxidation of α-chitin is the key factor affecting the resultant weight ratio of water-insoluble fractions, shape, length, and width of the chitin nano-whiskers obtained. The addition of 5.0 mmol of NaClO per gram of chitin in the oxidation seems to be optimum in terms of the preparation of mostly individualized nano-whiskers with high aspect ratios. Additional and various functionalities may be further provided to the TEMPO-oxidized chitin nano-whiskers by modification of the structure of the carboxylate groups, which are abundantly present on the surfaces.

2.4 Conclusions

The formation of C6 carboxylate groups in chitin can be controlled by controlling the amount of NaClO added in the TEMPO-mediated oxidation of α-chitin. Particularly, when 5.0 mmol of NaClO per gram of chitin is used, the weight percentage of the water-insoluble fraction in the TEMPO-oxidized chitin maintains as high as 90%, and the carboxylate content reaches 0.48 mmol/g. Besides, no N-deacetylation occurs on the TEMPO-oxidized chitins, irrespective of the amount of NaClO added in the oxidation. Moreover, the crystal structure and high crystallinity of the original α-chitin are maintained during TEMPO-mediated oxidation, showing that the C6 carboxylate groups formed are present only on the chitin crystallite surfaces. When this TEMPO-oxidized chitin is subjected to ultrasonic treatment in water, mostly individualized chitin nano-whiskers are obtained, and the dispersion is translucent. The average nano-whisker length and width were 340 and 8 nm, respectively, for the TEMPO-oxidized chitin.

2.5 References

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Chapter 3
TEMPO-Mediated Oxidation of β-Chitins to Prepare Chitin Nano-Fibers

3.1 Introduction

When the TEMPO-mediated oxidation with sufficient amounts of NaClO used as the primary oxidant is applied to crab shell α-chitins, the corresponding water-soluble polyuronic acid Na salts can be obtained by the regioselective oxidation of the C6 primary hydroxyl groups to carboxylate groups.[1,2] On the other hand, in the chapter 2, when the amount of NaClO added was controlled or reduced, water-insoluble fractions of the TEMPO-oxidized α-chitins are obtained in various yields, depending on the oxidation conditions. Translucent and homogeneous dispersions can be obtained by mild disintegration of the water-insoluble TEMPO-oxidized α-chitins in water, when they are prepared under specifically designed conditions. The dispersions consist of nano-whiskers with average width and length of 8 nm and 340 nm, respectively. The driving force to give TEMPO-oxidized α-chitin nano-whiskers is electrostatic repulsion and/or osmotic effect between anionically charged chitin fibrils of TEMPO-oxidized α-chitins in a similar manner to those for TEMPO-oxidized cellulose nano-fibrils.

Although most natural chitins have the α-type crystal structure, β-type chitin is present in squid pens and tubeworms and diatom spine.[3,4] In this chapter, therefore, TEMPO-mediated oxidation was applied to tubeworm and squid pen β-chitins to form C6 carboxylate groups just on the fibril surfaces and to obtain water-insoluble oxidized products in high yields by controlling the oxidation conditions. The water-insoluble fractions, when obtained, may have potential to be converted to individual and highly crystalline nano-fibers by mild disintegration in water.

3.2 Materials and Methods

3.2.1 Materials. Tubeworm (*Lamellibrachia satsuma*) and pens of squid (*Todarodes pacificus*) were used as resources of highly and low crystalline β-chitin, respectively. Professor Masahisa Wada in The University of Tokyo kindly provided Tubeworm samples, and Dainichi Seika Company in Japan provided squid pen. The β-chitins were purified, according to the conventional procedure,[5] and stored as never-dried samples at 4°C before use. The crystallinity indices of the tubeworm and squid pen β-chitins, calculated from X-ray diffraction patterns (see the following analysis section), were 0.74 and 0.51, respectively. The degrees of N-acetylation of tubeworm and squid pen β-chitins, which were calculated from their carbon and nitrogen contents were 0.99 and 0.90, respectively. TEMPO, sodium bromide, 2 M sodium hypochlorite solution, and other chemicals and solvents were of laboratory grade (Wako Pure Chemicals, Co., Japan), and used without further purification.

3.2.2 TEMPO-mediated oxidation. TEMPO-mediated oxidation was carried out to the purified and never-dried β-chitins, basically according to the same procedure as for crab shell α-chitin (see page 11, "2.2.2 TEMPO-mediated oxidation", in chapter 2).

3.2.3 Analyses of the water-insoluble fractions of TEMPO-oxidized β-chitins. The C6 carboxylate contents of the TEMPO-oxidized chitins were determined from conductivity titration curves and contents of the C2 amino groups determined beforehand by FT-IR method (See page 12, "2.2.3 Determination of carboxylate and aldehyde content", in chapter 2).

The freeze-dried chitin samples were converted to pellets using a disk apparatus for IR measurement, and subjected to X-ray diffraction analysis from 5° to 35° of diffraction angle 2θ using the reflection method by means of a Rigaku RINT 2000 with a Ni-filtered Cu Kα radiation (λ 0.15418 nm) at 40 kV and 40 mA. The crystallinity indices of β-chitins based on the 2θ scan from 5° to 35° were calculated accorded to the method reported by Li et al.,[6] which requires separation of the crystalline peaks from the diffuse background. The crystal size of the (010) plane was measured from the full width at half-height of the diffraction peak

(010) using Scherrer equation.[6]

Solid-state ^{13}C-NMR spectra of samples were recorded with a Bruker Avance spectrometer operated at 100 MHz for the ^{13}C nucleus. The spectra were acquired at room temperature with a 80 kHz proton dipolar decoupling field, matched cross-polarization (CP) fields of 80 kHz, a proton 90° pulse of 2.5 μs and magic angle spinning (MAS) at a spinning rate of 12 kHz.[7] The repetition time was 4 s, and the average number of 20,000 scans was acquired for each spectrum.

FT-IR spectra of the original and TEMPO-oxidized chitins were recorded with 4 cm^{-1} resolution and 64 scans on a Nicolet Magna 860 (Madison, WI, USA) in the transmission mode. Here, the original β-chitins were converted to KBr disks for FT-IR analysis. The TEMPO-oxidized chitin/water dispersions were cast on a Teflon plate followed by drying overnight at 50°C. The dried films were then treated with 0.1 M HCl to convert the sodium carboxylate groups to free carboxyls followed by vacuum-drying at 50°C for 24 h. The dried thin films were subjected to FT-IR analysis.

3.2.4. Disintegration of the water-insoluble TEMPO-oxidized β-chitins in water. The water-insoluble fraction of the TEMPO-oxidized β-chitin (never-dried form) was suspended in water at 0.2% consistency. The slurry (10 ml) was first disintegrated by a blender-type homogenizer at 15,000 rpm for 2 min (Excel Auto EO-4, Nihonseiki, Japan), and then treated by an ultrasonic homogenizer at 19.5 kHz and the 300 W output power (7 mm in the probe tip diameter, US-300T, Nihonseiki, Japan) for 1 min at room temperature.

3.2.5 Analyses of the TEMPO-oxidized β-chitin dispersions. ζ-Potentials of β-chitin components dispersed in water at 0.01% (w/v) consistency were measured at 20°C using a laser-Dopplerelectrophoresis-type apparatus (DTS5300 Zetasizer 3000, Malvern Instruments, UK).

The transmittances of the 0.1% TEMPO-oxidized chitin dispersions were measured from 300 to 700 nm using a Shimadzu UV-vis spectrophotometer (UV-1700).

A 10 μl aliquot of the 0.02% (w/v) TEMPO-oxidized chitin/water dispersion was mounted on a glow-discharged carbon-coated electron microscopy grid. After drying, the

sample grid was observed at 100 kV using a JEOL transmission electron microscope (JEM 2000-EXII). The fibril images were taken at diffraction contrasts in the bright-field mode using a low-dose exposure, and the micro-diffraction technique was used in some cases.[8] All micrographs were collected with a CCD camera (Keen View, Olympus Soft Imaging Solutions, Germany) and recorded using iTEM® software provided by the CCD camera company.

3.3 Results and Discussion

3.3.1 Carboxylate and aldehyde groups in the TEMPO-oxidized β-chitins. Figure 3.1 shows the relationships between the amount of NaClO added as the primary oxidant in the TEMPO-mediated oxidation and either weight ratio of the water-insoluble fraction or its carboxylate and aldehyde contents for tubeworm and squid pen β-chitins. The weight ratios of the water-insoluble fractions were decreased with increasing the amount of NaClO added. All water-soluble fractions consisted of chitouronic acids, whose C6 positions were completely oxidized to carboxylate groups, which were confirmed by their solution-state ^{13}C-NMR spectra.[1,3] Carboxylate contents in the water-insoluble fractions were increased with increasing the NaClO addition level up to about 5 mmol NaClO per gram of chitin, and then roughly reached plateau levels for both tubeworm and squid pen β-chitins. Thus, the weight ratios of the water-insoluble fractions and their carboxylate contents can be controlled to some extent by controlling the amount of NaClO added in the TEMPO-mediated oxidation.

For example, when the NaClO addition level was 10.0 mmol/g, the weight ratios of the water-insoluble TEMPO-oxidized tubeworm and squid pen β-chitins were 71% and 49%, respectively. The contents of total oxidized groups (carboxylate + aldehyde groups) were 0.25 and 0.36 mmol/g, respectively. The higher crystallinity and larger crystal size (or smaller surface area) of tubeworm β-chitin might have resulted in such higher weight ratio of the water-insoluble fraction and smaller amounts of oxidized groups than for the squid pen β-chitin.

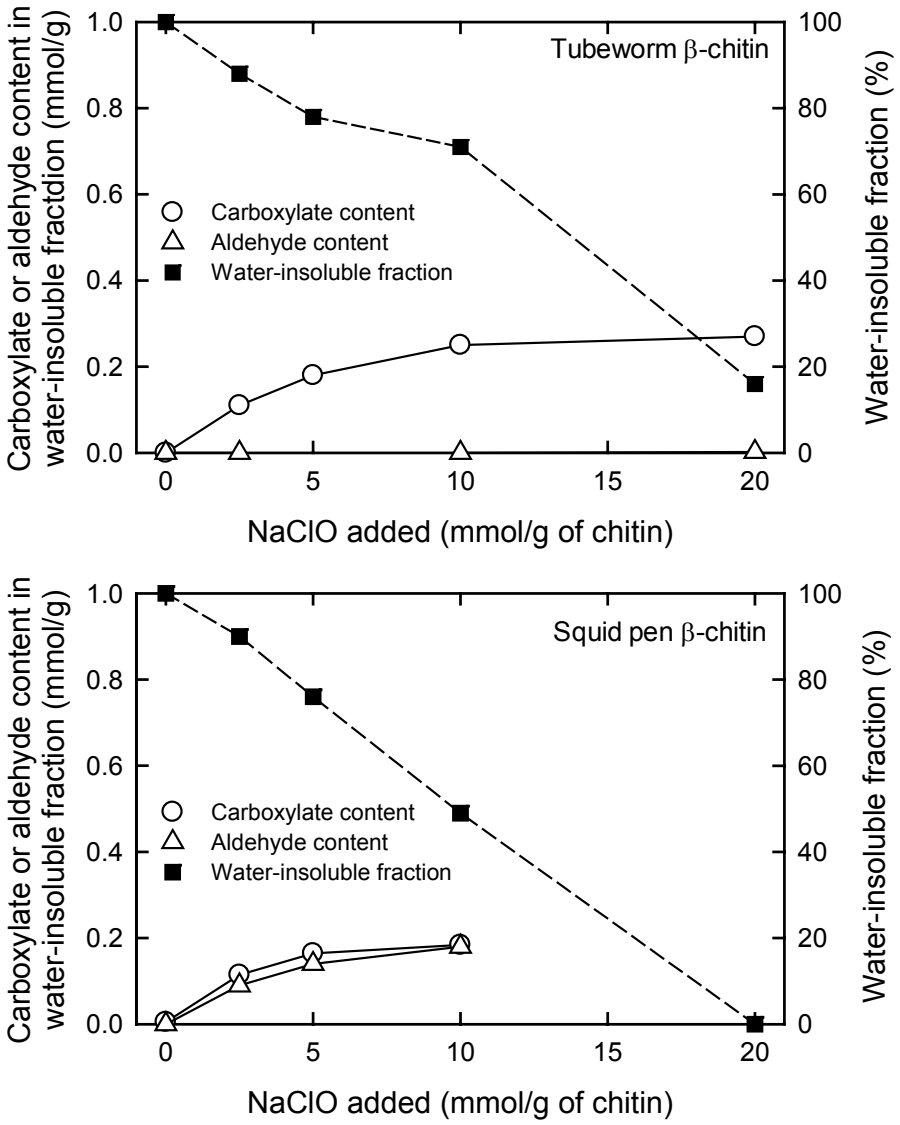

Figure 3.1. Relationship between the NaClO addition level in the TEMPO-mediated oxidation of tubeworm and squid pen β-chitins and either weight ratio of water-insoluble fraction or its carboxylate and aldehyde contents.

It is noteworthy that nearly no aldehyde groups were present in the TEMPO-oxidized tubeworm β-chitins, whereas both carboxylate and aldehyde groups were formed in similar ratios in the TEMPO-oxidized squid pen β-chitins. Thus, the aldehyde groups are likely to be formed in relatively disordered regions of squid pen β-chitins, and to present in the TEMPO-oxidized products forming hydrate or hemiacetal structures, which are highly resistant to further oxidation to carboxylate groups during the TEMPO-oxidation. On the other hand, no such aldehyde groups remain in the highly crystalline tubeworm β-chitin. This hypothesis may be applicable also to TEMPO-oxidized native celluloses, which have various aldehyde contents, depending on the cellulose origins.[9]

3.3.2 Changes in crystallinity and crystal size of β-chitins by TEMPO-mediated oxidation. X-ray diffraction patterns of the original and TEMPO-oxidized β-chitins are depicted in Figure 3.2, and the relationships between the NaClO addition level and either crystallinity index or crystal size of the (010) plane of β-chitins are plotted in Figure 3.3. The original crystal structures of β-chitins were basically maintained in the water-insoluble fractions during the TEMPO-mediated oxidation. The diffraction peak due to the hydrate (010) space, which was originally present in the squid pen β-chitin around 2θ 9° but not in the original tubeworm β-chitin,[10,11] appeared in the TEMPO-oxidized tubeworm β-chitins.

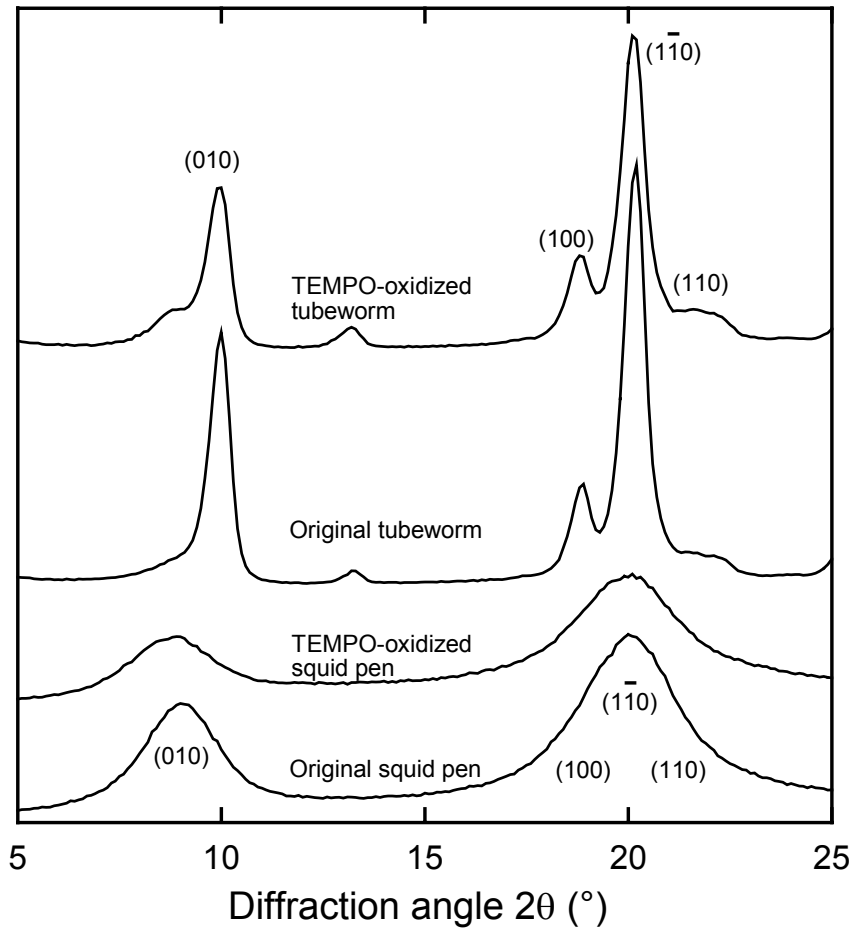

Figure 3.2. X-ray diffraction patterns of the original β-chitins and their water-insoluble fractions of TEMPO-oxidized products prepared with 10 and 5 mmol NaClO per gram of tubeworm and squid pen β-chitins, respectively.

Crystallinity indices were decreased for both β-chitins with increasing the NaClO addition level; disordered regions are formed in the β-chitins to some extent by the oxidation.

On the other hand, the crystal sizes of the (010) plane determined from the peak widths of (010) were mostly maintained by the oxidation. These results indicate that almost all carboxylate and aldehyde groups formed in the water-insoluble TEMPO-oxidized β-chitins are selectively present on the crystallite surfaces of β-chitins. Based on the results in Figures 3.1 and 3.3, therefore, the remarkable decrease in the weight ratio of the water-insoluble fraction prepared with 20 mmol NaClO per gram of tubeworm β-chitin or with 10 mmol NaClO per gram of squid pen β-chitin in the oxidation might have been caused by significant decreases in the fibril lengths, not widths, of the original β-chitins during the TEMPO-mediated oxidation at these high NaClO addition levels. This subject will be further discussed later.

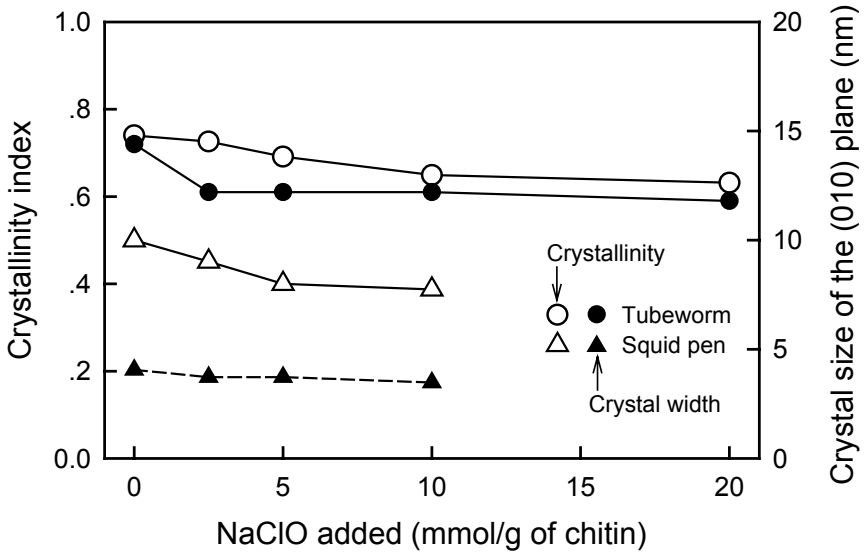

Figure 3.3. Changes in crystallinity and crystal size of the water-insoluble fractions of TEMPO-oxidized β-chitins prepared with various amounts of NaClO.

3.3.3. Chemical structures of TEMPO-oxidized β-chitins. Figures 3.4 and 3.5 show solid-state ^{13}C-NMR and FT-IR spectra, respectively, of the original β-chitins and the water-insoluble fractions of the TEMPO-oxidized β-chitins prepared with NaClO of 5 or 10 mmol/g. The patterns of the original solid-state ^{13}C-NMR spectra were essentially unchanged even after the TEMPO-mediated oxidation. When the relative signal areas of C6 primary hydroxyls around 60 ppm to those of C1 around 103 ppm are compared before and after the oxidation, about 10-14% of the original signal areas due to the C6 primary hydroxyls decreased by the oxidation for both tubeworm and squid pen β-chitins. Inversely, the relative signal areas due to C=O signals around 176 ppm increased in both cases. Hence, the ^{13}C-NMR results support that the carboxylate groups formed by the TEMPO-mediated oxidation originate from the C6 primary hydroxyls in the β-chitins. Moreover, the relative signal areas around 23 ppm due to acetyl amide carbons to those due to C1 carbons were nearly unchanged after the TEMPO-oxidation for both tubeworm and squid pen β-chitins, showing that no N-deacetylation occurred at the C2 positions of β-chitins during the oxidation.

FT-IR spectra also support the above conclusions. Carboxyl groups are formed in the β-chitins by the TEMPO-mediated oxidation, although the original crystal structures and degrees of N-acetylation are maintained.[12,13]

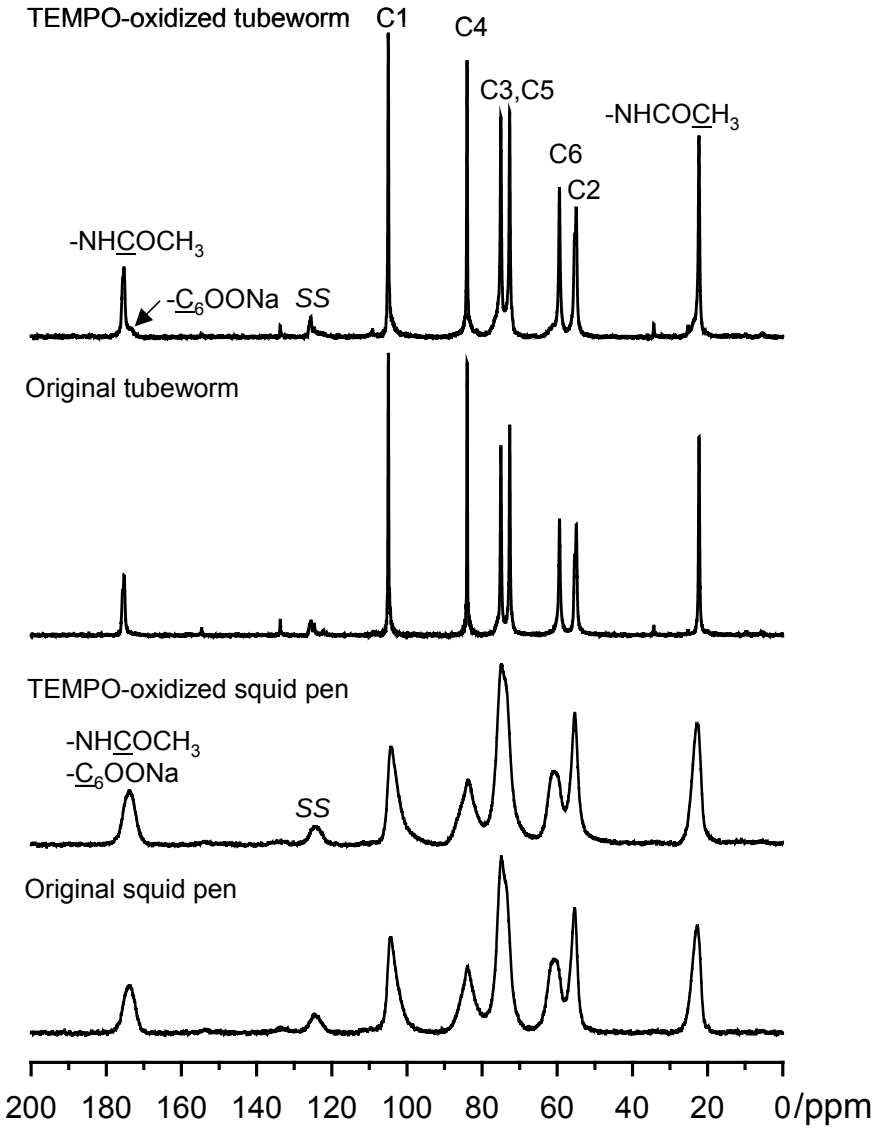

Figure 3.4. Solid-state ^{13}C-NMR spectra of the original β-chitins and their water-insoluble fractions of the TEMPO-oxidized products prepared with 10 and 5 mmol NaClO per gram of tubeworm and squid pen β-chitins, respectively. *SS*: spinning side band.

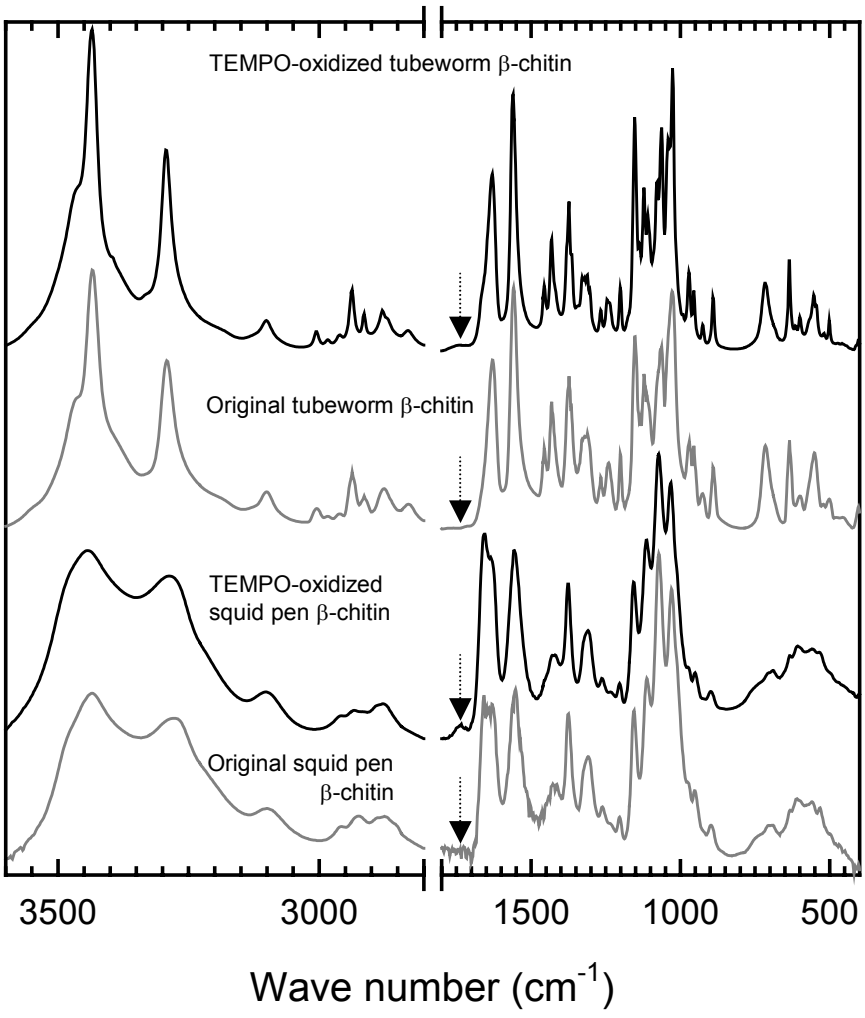

Figure. 3.5. FT-IR spectra of the original β-chitins and their water-insoluble fractions of TEMPO-oxidized products prepared with 10 and 5 mmol NaClO per gram of tubeworm and squid pen β-chitins, respectively. Arrows show the C=O band position due to carboxyl groups.

3.3.4. Disintegration of the water-insoluble TEMPO-oxidized β-chitins in water. The water-insoluble TEMPO-oxidized tubeworm and squid pen β-chitins as well as the corresponding original β-chitins were disintegrated in water. As shown in Figure 3.6, the original squid pen β-chitin and its TEMPO-oxidized products prepared under any conditions could not be converted to homogeneous dispersions even after extended disintegration time. Probably because numerous polyion complexes are formed between the amino groups present in the original squid pen β-chitin and the C6 carboxylate groups formed by the oxidation, individualization of fibrils could not be achieved in this case. The significant amounts of aldehyde groups formed by the oxidation and present in the water-insoluble TEMPO-oxidized squid pen β-chitins (Figure 3.1) might also have caused such resistance to the conversion to individual fibrils, forming inter-fibrillar hemiacetal linkages between aldehyde groups formed and hydroxyls originally present in the β-chitin.

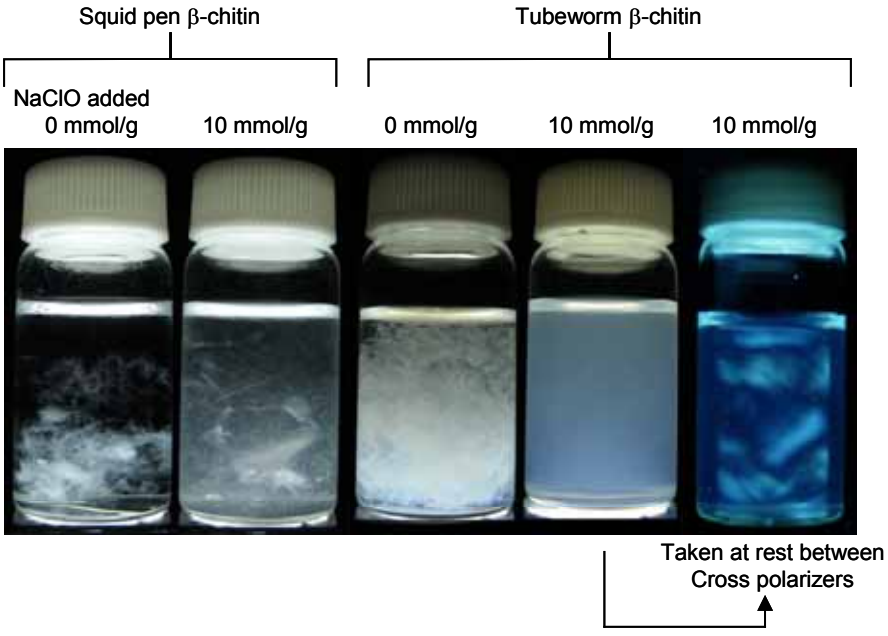

Figure 3.6. Photographs of suspensions or dispersions of the original and TEMPO-oxidized β-chitins in water at 0.2% consistency.

On the other hand, the water-insoluble fraction of the TEMPO-oxidized tubeworm β-chitin prepared with 10 mmol NaClO per gram of chitin homogeneously dispersed in water, providing a highly viscous and translucent gel (Figure 3.6). The dispersion showed birefringence between cross polarizers, indicating that dispersed fibrils having optical anisotropy were stably present in the bottle. ζ-Potentials of the TEMPO-oxidized tubeworm β-chitin components dispersed in water were around -60 mV, which is much negatively higher than the water suspensions of TEMPO-oxidized squid pen β-chitins (Figure 3.7).

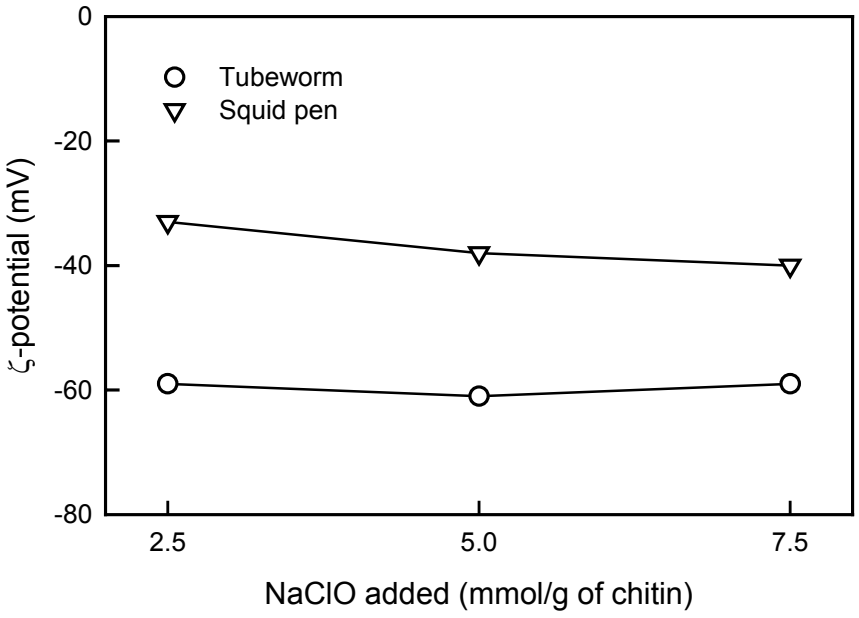

Figure 3.7. ζ-Potentials of the dispersions or suspensions of TEMPO-oxidized tubeworm and squid pen β-chitins in water at 0.01% consistency.

Thus, the TEMPO-oxidized tubeworm β-chitin components had highly anionic charges due to carboxylate groups formed by the oxidation, and caused such stably dispersed state in water by electrostatic repulsion between the components. Because the original tubeworm β-chitin has a quite high degree of N-acetylation (0.99) or a quite low amount of C2 primary amino groups, the surface charges of the tubeworm β-chitin fibrils are governed by anionic charges due to the C6 carboxylate groups formed by the TEMPO-mediated oxidation rather than the amino groups slightly and originally present in the tubeworm β-chitin. Hence, the homogeneously dispersed state was obtained by disintegration of the water-insoluble TEMPO-oxidized tubeworm β-chitin in water, which was different from the results of the TEMPO-oxidized squid pen β-chitins.

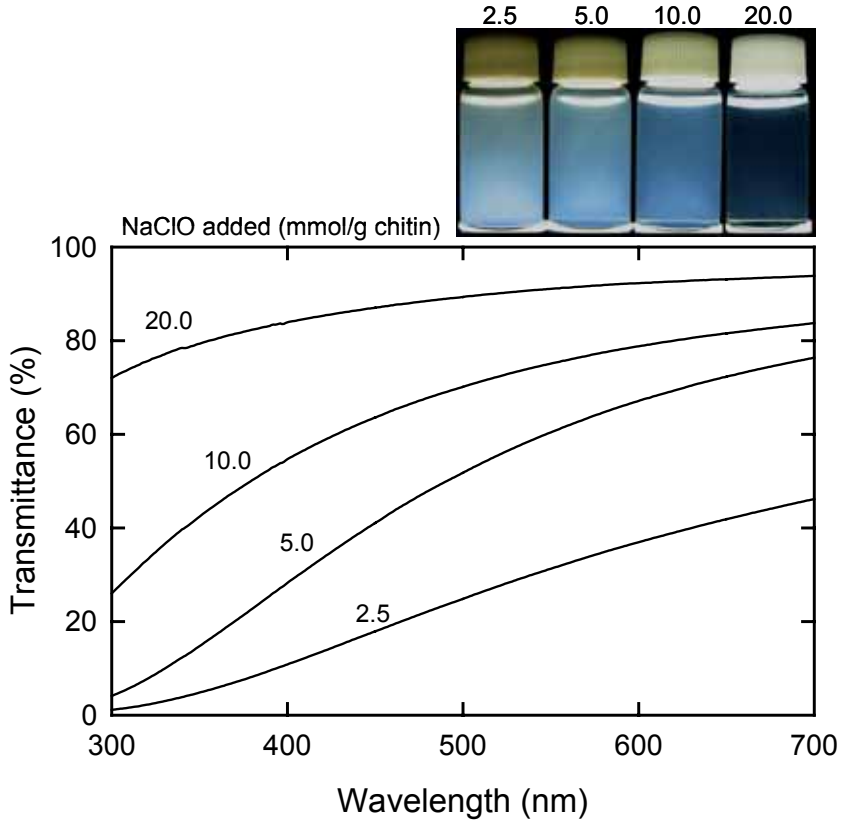

Figure 3.8. UV-visible light-transmittance spectra for the 0.1% aqueous dispersions of TEMPO-oxidized tubeworm β-chitins prepared with different NaClO addition levels and photographs of the corresponding dispersions.

Light transmittances of the 0.1% dispersions for the water-insoluble fractions of the TEMPO-oxidized tubeworm β-chitins prepared with different amounts of NaClO are shown in Figure 3.8 together with their photographs. The light transmittance was increased as the NaClO addition level increased, showing that individualization of the tubeworm β-chitin fibrils was enhanced by the increased amounts of carboxylate groups formed in the water-insoluble TEMPO-oxidized products. Although the dispersion for the TEMPO-oxidized tubeworm β-chitin prepared with 20 mmol NaClO per gram of chitin was highly transparent, the yield of the water-insoluble fraction was only about 16% in this case (Figure 3.1). Thus, the TEMPO-oxidized tubeworm β-chitins prepared with either 5.0 or 10.0 mmol NaClO per gram of chitin are suitable for preparation of homogeneously dispersed and translucent (not transparent) gels from the water-insoluble fractions by disintegration in water.

3.3.5. TEM observation of TEMPO-oxidized tubeworm β-chitin nano-fibers. Transmission electron microphotographs (TEM) of the dispersion of the water-insoluble fraction of TEMPO-oxidized tubeworm β-chitin prepared with 10 mmol NaClO per gram of chitin are observed (Figure 3.9). Long TEMPO-oxidized tubeworm β-chitin nano-fibers were clearly observed, and had widths ranging 20-50 nm and lengths of more than several microns. Thus, although the dispersions were not completely transparent but only translucent, the tubeworm β-chitin fibrils were mostly converted to highly crystalline and individual nano-fibers dispersed in water by the TEMPO-mediated oxidation and the following mechanical disintegration.

As shown by one of the white arrows in the upper TEM image of Figure 3.9, the fibril had a twisted part, indicating that each fibril has a flat and ribbon-like morphology 20-50 nm in width and *ca.* 15 nm in thickness. Another white arrow in Figure 3.9 shows an edge of the nano-fiber probably formed by scission during disintegration in water. Because each individual nanofibril in Figure 3.9 had a clear electron diffraction spots at any locations, one TEMPO-oxidized tubeworm β-chitin nano-fiber does not consist of multiple elementary fibrils but forms a perfect crystallite.

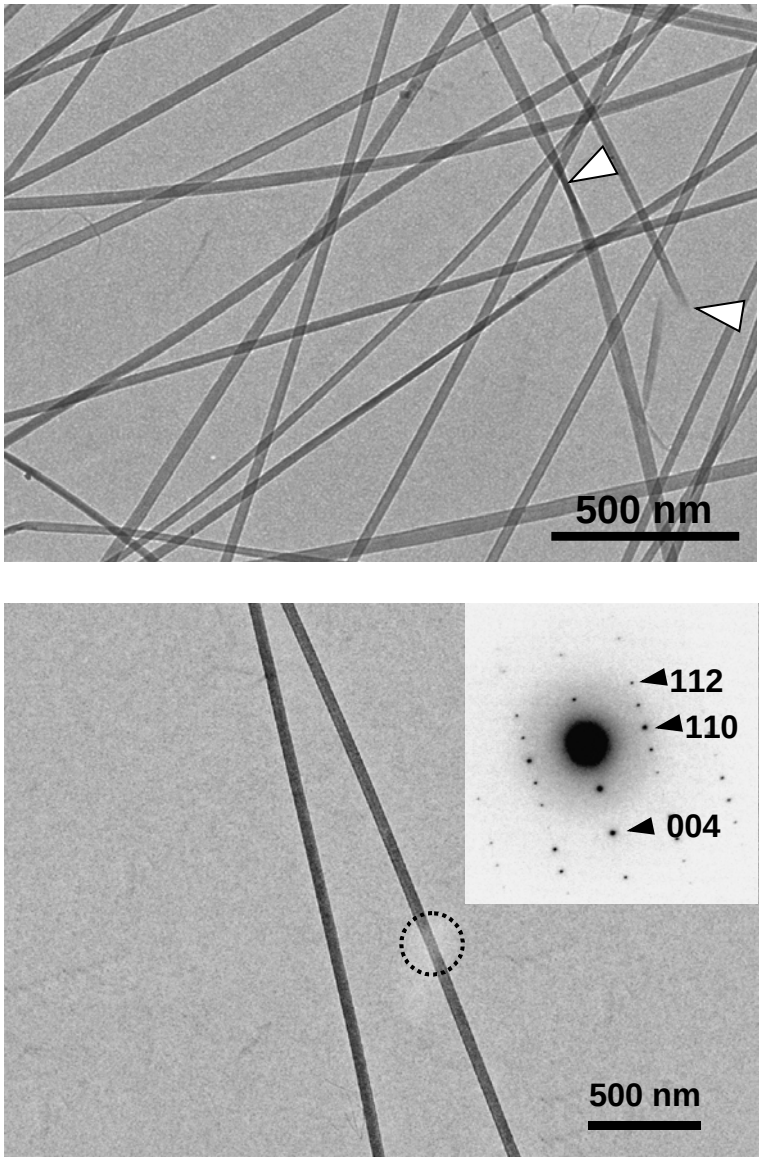

Figure 3.9. Transmission electron microphotographs and electron diffraction diagram of nano-fibers of TEMPO-oxidized tubeworm β-chitin prepared with 10 mmol NaClO per gram of chitin. White arrows show twisted and cut parts of the fibrils.

The fibril width was determined from TEM images, and the distribution of nanofiber widths from TEMPO-oxidized tubeworm β-chitins was shown in Figure 3.10. Even though the fibril widths determined from TEM images had large deviations, the decreasing pattern of the fibril width with increasing the NaClO addition level was observed.

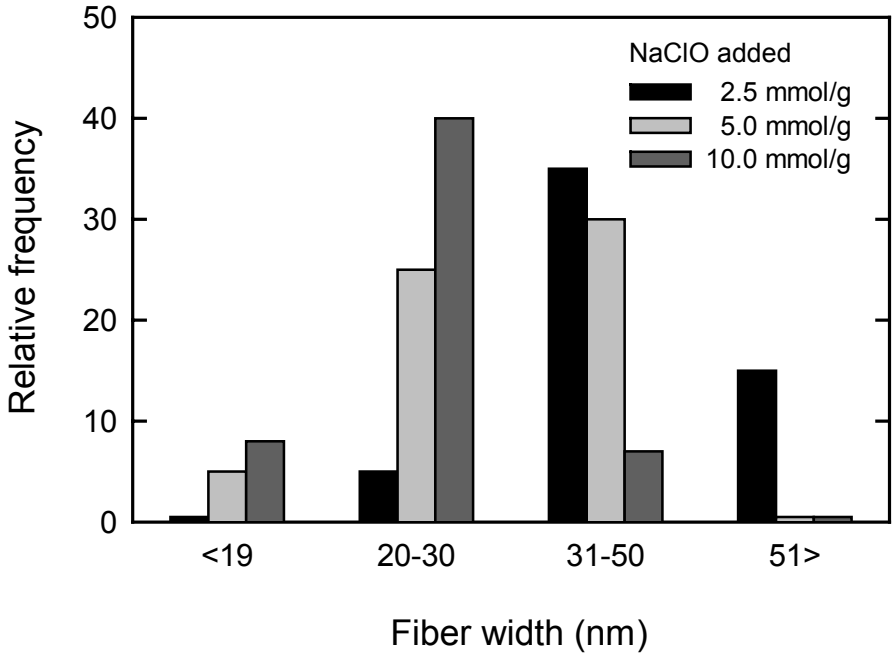

Figure 3.10. Width distribution of chitin nano-fibers prepared from tubeworm β-chitin by TEMPO-mediated oxidation determined from TEM images.

Figure 3.11 shows changes in the weight ratio of the water-insoluble fraction from Figure 3.1, the fibril width determined from TEM images from Figure 3.10, and the crystal sizes of

the $(1\bar{1}0)$ and (010) planes determined from X-ray diffraction patterns in Figure 3.2 for the TEMPO-oxidized tubeworm β-chitins prepared with different NaClO addition levels.

The decreasing pattern of the fibril width with increasing the NaClO addition level, determined from TEM images, is correlated well to that of the weight ratio of the water-insoluble fraction in the range of 2.5-10 mmol NaClO per gram of the tubeworm β-chitin. The average widths were 39.6 nm, 30.0 nm and 26.4 nm, when the TEMPO-oxidized β-chitins were prepared with NaClO of 2.5 mmol, 5.0 mmol and 10 mmol per gram of chitin, respectively. At least in the NaClO addition range of 2.5-10 mmol/g, the oxidation of chitin molecules to water-soluble chitouronic acids takes place from each edge of the fibril width to inside crystallites. These chitouronic acid molecules formed are dissolved in the aqueous oxidation media, and removed from the fibril surfaces like peeling-off behavior during the TEMPO-mediated oxidation, resulting in the decrease in the fibril width with increasing the NaClO addition.

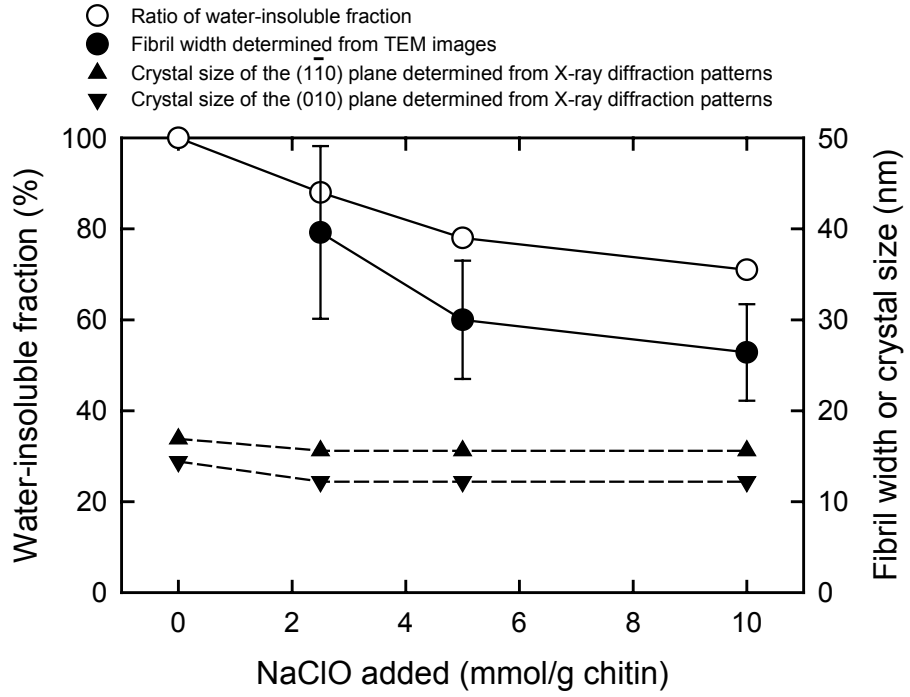

Figure 3.11. Changes in the weight ratio of water-insoluble fraction, fibril width determined by TEM images and crystal sizes determined by X-ray diffraction patterns for the TEMPO-oxidized tubeworm β-chitin by the NaClO addition level.

However, the fibril widths of the TEMPO-oxidized tubeworm β-chitin nano-fibers observed by TEM in Figure 3.9 and 3.10 were always larger than the $(1\bar{1}0)$ and (010) crystal sizes determined from X-ray diffraction patterns of the water-insoluble TEMPO-oxidized tubeworm β-chitins. Moreover, the crystal sizes were nearly unchanged to the NaClO addition level in the range of 2.5-10 mmol/g; the decreasing pattern of fibril width determined from TEM images did not correlated to the patterns of crystal sizes. As described previously, the TEMPO-oxidized tubeworm β-chitin nano-fibers have flat and ribbon-like morphologies. Probably, only the diffraction planes parallel to the surfaces of the ribbon-like TEMPO-oxidized tubeworm nano-fibers observed in Figure 3.9 are detectable as X-ray diffraction peaks. This is because the flat and ribbon-like nano-fibers are packed in parallel manner to the surface of the sample pellets prepared by pressing and subjected to the reflection-type X-ray diffraction analysis. X-ray diffraction can provide only information to the thickness direction of the flat and ribbon-like nano-fibers, while TEM images provide the width information of the nano-fibers. Thus, the discrepancy between the fibril widths determined from TEM images and the crystal sizes determined by X-ray diffraction patterns in Figure 3.11 is likely to be caused by the flat and ribbon-like morphology of tubeworm β-chitin fibrils.

Based on the above results, chitin molecules in the flat and ribbon-like fibrils are oxidized from each edge of the fibril width in Figure 3.9 to inside crystallites, forming water-soluble chitouronic acid molecules, as the NaClO addition level increases in the TEMPO-mediated oxidation. Consequently, the width of the fibrils decreases with the NaClO addition. On the other hand, the thickness of the fibrils is maintained during the oxidation; chitin molecules are not removed as chitouronic acids from the upper or lower sides of the flat and ribbon-like fibrils in Figure 3.9.

3.4 Conclusions

In the case of squid pen β-chitin, although the water-insoluble fractions were obtained from the TEMPO-oxidized products by controlling the NaClO addition level, the oxidized products could not be converted to individual nano-fibers under any conditions examined so far by disintegration in water. On the other hand, when tubeworm β-chitin was oxidized by

the TEMPO-mediated system with 2.5-10 mmol NaClO per gram of chitin, the water-insoluble fractions with carboxylate contents of 0.18-0.25 mmol/g were obtained in the yields of more than 70%. X-ray diffraction, solid-state ^{13}C-NMR and FT-IR analyses revealed that the water-insoluble TEMPO-oxidized tubeworm β-chitins had crystallinity indices, crystal sizes and degrees of N-acetylation quite similar to those of the original β-chitin. Nearly no aldehyde groups were present in the water-insoluble fractions. Homogeneous, highly viscous and translucent (not transparent) gels can be obtained by disintegration of the above water-insoluble TEMPO-oxidized tubeworm β-chitins in water. The gels consist of nano-fibers 20-50 nm in width and at least several microns in length. Thus, the carboxylate groups formed by the TEMPO-mediated oxidation are likely to be present selectively on the tubeworm β-chitin fibril surfaces. TEM images of the TEMPO-oxidized tubeworm showed that each fibril had the flat and ribbon-like morphology. As the NaClO addition level increased, the widths of nano-fibers were decreased by peeling-off of chitin molecules as water-soluble chitouronic acid molecules in the aqueous oxidation media. When sufficient amounts of NaClO were used, both tubeworm and squid pen β-chitins are mostly converted to water-soluble chitouronic acid Na salts by complete oxidation of the C6 primary hydroxyls to carboxyl groups.

3.5 References

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Chapter 4
Squid Pen β-Chitin Nano-Fibers Prepared by Mechanical Treatment under Acid Conditions

4.1 Introduction

Highly crystalline cellulose nanofibers completely individualized in water can be prepared by 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO)-mediated oxidation of native celluloses followed by mild mechanical treatment in water.[1,2] TEMPO-mediated oxidation is also applicable to crab shell α-chitins and tubeworm β-chitins, and chitin nano-whiskers or nano-fibers, respectively, have been successfully and quantitatively obtained. In both cases of cellulose nano-fibers and chitin nano-whiskers or nano-fibers, the selective formation of C6 carboxylate groups from the C6 primary hydroxyl groups on cellulose I and chitin crystallite surfaces by TEMPO-mediated oxidation is the key point to prepare individualized nano-fibers or nano-crystals by successive mechanical treatments in water.[2] When a sufficient amount of carboxylate groups is formed by TEMPO-mediated oxidation, transparent (or translucent) and highly viscous dispersions of cellulose nano-fibers or chitin nano-whiskers/nano-fibers can be obtained by electrical repulsion and/or osmotic effect between the anionically charged nano-fibers during mild mechanical treatment in water. However, because the cellulose nanofibers and chitin nano-whiskers/nano-fibers prepared from the corresponding TEMPO-oxidized natural polysaccharides are chemically modified celluloses and chitins, respectively, a number of safety issues need to be addressed before either of these can be used practically as medical materials. Moreover, due to the low crystallinity and degree of N-acetylation of squid pen β-chitins, although the water-insoluble fractions were obtained from the TEMPO-oxidized products by controlling the NaClO addition level, the oxidized products could not be converted to individual nano-fibers under any conditions examined so far by disintegration in water.

On the other hand, isolated and purified α- and β-chitins originally have small amounts of

the C2 amino group,[3,4] which can be cationized under acid conditions. Based on the aforementioned mechanisms to prepare cellulose nano-fibers and α-chitin nano-whiskers or tubeworm β-chitin nano-fibers by TEMPO-mediated oxidation, α- and β-chitins may have potential to be converted to chitin nano-fibers by cationization of the C2 amino groups with acid followed by disintegration under acid conditions, without any chemical modification of the chitins. Here, a sufficient number of cationic C2 ammonium groups must be present on the chitin crystallite surface.

In this chapter, therefore, we studied the preparation of individualized chitin nano-fibers dispersed in water by simple mechanical treatment of chitins under acid conditions, where the C2 amino groups in the chitins are cationized.

4.2 Materials and Methods

4.2.1 Materials. Two α-chitins and two β-chitins were used in this study. The α-chitin sample originating from crab shell was a commercial product (Wako Pure Chemicals, Co., Japan) and used without further purification. Crab tendon (*Chionoecetes opilio*), tubeworm (*Lamellibrachia satsuma*), and squid pen (*Todarodes pacificus*) were purified to obtain the chitins according to the conventional procedure and stored as never-dried chitins at 4°C before use. The yield of purified squid pen chitin was 31%, based on the dry weight of the starting unpurified squid pen. The crystallinity indices of crab shell and crab tendon α-chitins and squid pen and tubeworm β-chitins, which were determined by X-ray diffraction analysis,[7] were 0.57, 0.61, 0.51, and 0.74, respectively (Figure 4.1). The degrees of N-acetylation of these α- and β-chitins, which were calculated from their carbon and nitrogen contents, determined by elementary analysis, were 0.93, 0.93, 0.90, and 1.00, respectively.

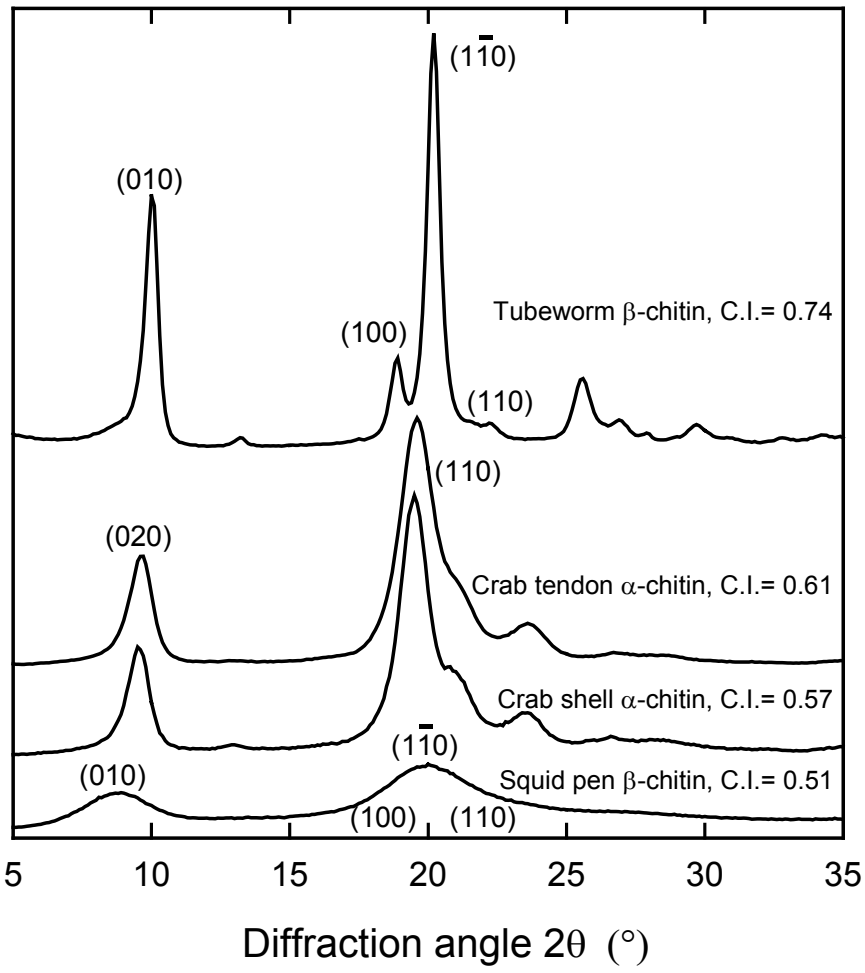

Figure 4.1. X-ray diffraction patterns and crystallinity indices (C.I.) of crab shell and crab tendon α-chitins and squid pen and tubeworm β-chitins used in this study.

4.2.2 Mechanical disintegration. The wet chitins were suspended in water at 0.1-0.3% consistency. Several drops of acetic acid, a dilute hydrochloric acid solution, or a dilute NaOH solution were added to the chitin slurries to adjust their pH values from 3 to 8.

Ultrasonication was applied to the slurries (15 mL each) for 2 min using an ultrasonic homogenizer at 19.5 kHz and the 300 W output power (7 mm in the probe tip diameter, US-300T, Nihonseiki, Japan). The temperature increase was below 5°C during the ultrasonication.

4.2.3 Optical transmittance. A chitin dispersion was introduced into a poly (methyl methacrylate) disposable cuvette, and the transmittance was measured from 300 to 700 nm using a Shimadzu UV-vis spectrophotometer (UV-1700). The spectrum of a cuvette filled with water was used as a reference to correct the transmittance of the slurry or dispersion.

4.2.4. Microscopic observations. Slurries of the chitins in water before ultrasonication were observed using an optical microscope (Olympus BX50) equipped with a phase-contrast lens (Olympus UPlanFLN-PH), cross-polarizers, and a digital camera (Olympus PD20). The transmission electron micrographs (TEM images) were collected with a charge coupled device (CCD) camera and recorded using iTEM® software. The details were described in page 14, "2.2.8 Microscopic observations", in chapter 2.

4.2.5 X-ray diffraction analysis. The original chitins and chitin/acidic water dispersions were freeze-dried. The dried chitin (0.1 g) was converted to a pellet 1 cm in diameter by pressing at about 750 MPa for 1 min using a disk apparatus for IR measurement, and subjected to X-ray diffraction analysis. Details were described in page 32, "3.2.3 Analyses of the water-insoluble fractions of TEMPO-oxidized β-chitins", in chapter 3.

4.2.6 FT-IR spectroscopy. Thin films were prepared by casting the purified chitin/water slurries or their dispersions after ultrasonication at pH 4 on a Teflon plate and then drying overnight at 50°C, and subjected to FT-IR measurement according to the procedure described in page 13, "2.2.7 FT-IR spectroscopy", in chapter 2.

4.2.7 ζ-Potential measurement. ζ-Potentials of chitin nano-fibers dispersed in water were measured at 20°C using a laser-Dopplerelectrophoresis-type apparatus (DTS5300 Zetasizer 3000, Malvern Instruments, U.K.).[5] The consistency of chitin nano-fibers in water

was set to 0.01% (w/v), and the pH value changed from 3 to 7.

4.2.8 Preparation of squid pen β-chitin nano-fibril films and their analyses. The squid pen β-chitin nano-fibril films were prepared according to the reported method to prepare TEMPO-oxidized cellulose nano-fiber (TOCN) films.[6] An approximately 0.1% squid pen β-chitin nano-fibril/acidic water dispersion was converted to a film with about 20 μm thickness on a surface-hydrophilized polytetrafluoroethylene (PTFE) membrane with 0.1 μm pore size (Advantec Toyo, Japan) by suction filtration. The films obtained were dried in a ventilated oven at 50°C overnight without forced air-flow. Light transmittances of the films were measured from 200 to 1000 nm wavelength using a Shimadzu UV-1700 UV-vis spectrometer, and were correlated based on their film thickness values using the Lambert-Beer's law. Tensile strengths and Young's moduli of the films 3 mm in width and at least 20 mm in length were measured at 1.0 mm min^{-1} and 10 mm span length using a Shimadzu EZ-TEST instrument equipped with a 500 N load cell.

4.2.9 Preparation of aerogels from squid pen β-chitin nano-fibrils and their SEM observation. Aerogels consisting of squid pen β-chitin nano-fibrils were prepared by freeze-drying of the squid pen β-chitin nano-fibril/acidic water dispersions at 0.1% consistency. Cross-sections of the aerogels were observed using a field-emission-type SEM (Hitachi S-4000) after platinum sputtering at 20 mA for 120 s.

4.3 Results and Discussion

4.3.1 Ultrasonication of α- and β-chitins in water at pH 3-8. We used crab shell and crab tendon as sources of α-chitin and squid pen and tubeworm as sources of β-chitin. The optical microphotographs of the four chitins used are shown in Figure 4.2 at two different magnifications. The crab shell and crab tendon α-chitins have particle and flake forms, respectively, while the squid pen and tubeworm β-chitins have heterogeneous film-like and fibrous shapes, respectively.

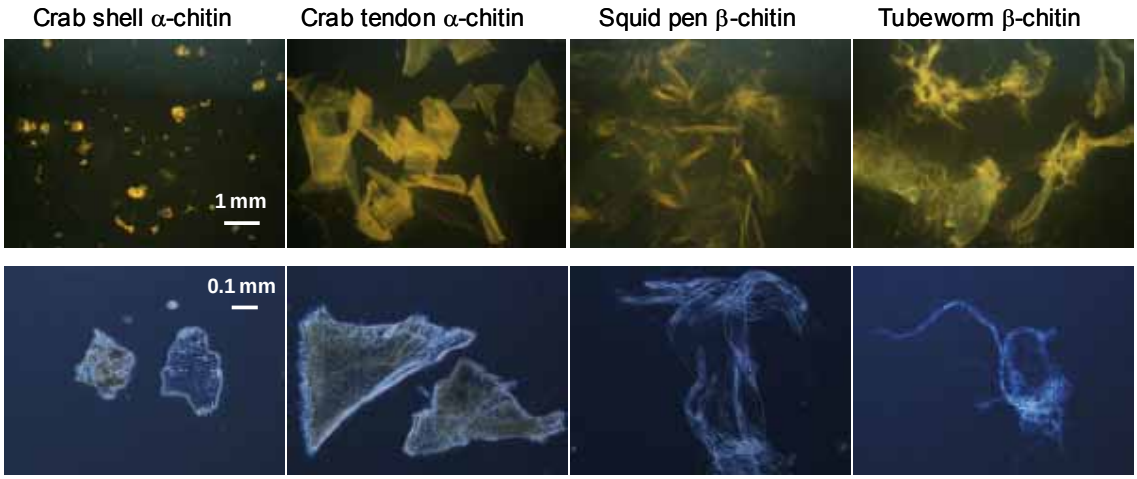

Figure 4.2. Optical microphotographs of crab shell and crab tendon α-chitins and squid pen and tubeworm β-chitins taken at two different magnifications with a polarized-light microscope.

A mixture of chitin (15 mg) and water (15 mL) was adjusted to the designated pH by adding acetic acid, dilute HCl, or dilute NaOH solution and then subjected to ultrasonication. The chitin/water dispersions thus prepared were placed motionlessly at least one day after the ultrasonication, and then the photos in Figure 4.3 were taken. The two α-chitin samples with particle and flake forms and the tubeworm β-chitin used in this study sedimented spontaneously at the bottom of the bottle without any dispersion or swelling in water under any pH value, even after extended ultrasonication. On the other hand, the β-chitin obtained from squid pen was dispersed well and converted to transparent and highly viscous suspensions in the pH range from 3 to 4 by ultrasonication. Both acetic acid and hydrochloric acid were available for the pH control to prepare transparent β-chitin dispersions.

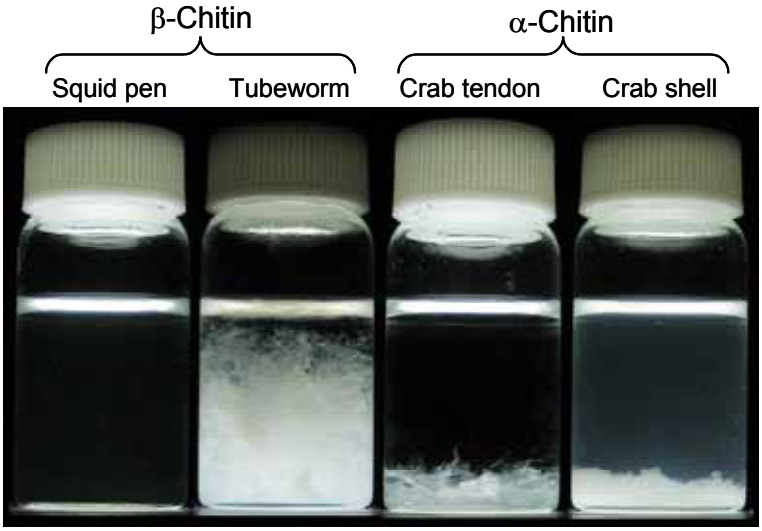

Figure 4.3. Photographs of dispersions or slurries of α- and β-chitins prepared by ultrasonication in water at pH 4 for 2 min.

Photographs of the dispersions of squid pen β-chitin in water at pH 3-8, and of the corresponding visible-light transmittances are shown in Figure 4.4. The transmittances of the dispersions prepared at pH 3 and pH 4 were over 85% for visible light, and the dispersion at pH 5 was slightly turbid. The original shape of squid pen in water was maintained at the pH values of more than 6. Thus, the pH adjustment of the squid pen β-chitin/water slurry at 3-4 is needed to convert it to transparent dispersions by the ultrasonication. Because the pKa of the C2 ammonium salt of the anhydroglucosamine unit in chitin is 6.3,[7] complete cationization of the C2 amino groups in the squid pen β-chitin at pH 3-4 is probably one of the necessary points to prepare the transparent chitin nanofiber/water dispersions.

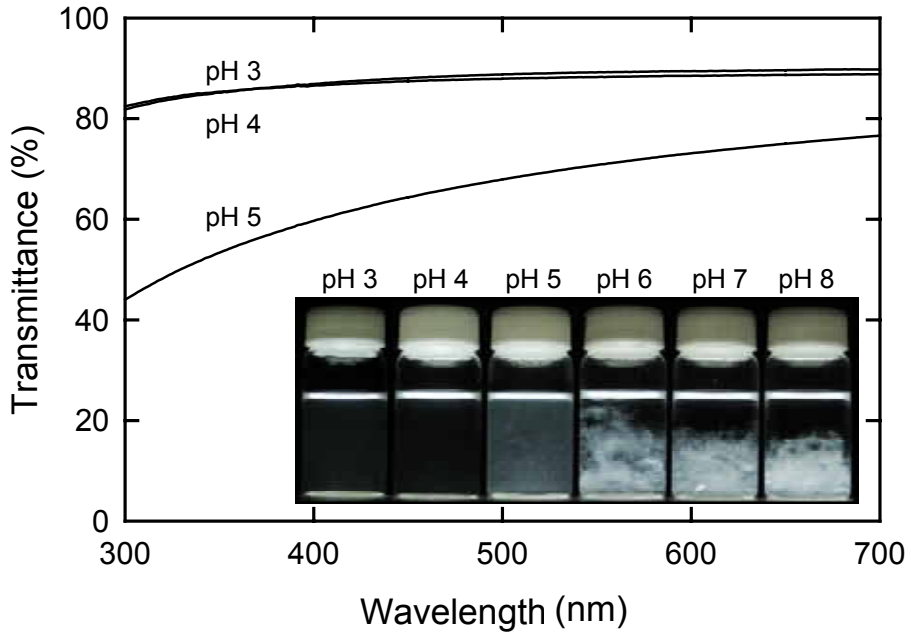

Figure 4.4. Photographs of dispersions and slurries of squid pen β-chitin prepared by ultrasonication in water at pH 3-8 for 2 min. The UV-visible light transmittance spectra for three of the six samples are also shown (10 mm path length).

The difference in dispersion behavior between squid pen and tubeworm β-chitins in Figure 4.3 may be due to that in the degree of N-acetylation between them. Because nearly no amino groups are present in tubeworm β-chitin, whose degree of N-acetylation is almost 1.0, no cationic charges are formed on tubeworm β-chitin in water, even at pH 3-4, resulting that no transparent dispersions in water were obtained under any ultrasonication conditions. On the other hand, two α-chitins have the potential to be cationized in water at pH 3-4, because their degrees of N-acetylation are 0.93. However, these α-chitins could not be converted to transparent dispersions under any ultrasonication conditions. As shown in Figure 4.1, the

squid pen β-chitin has a crystallinity index lower than those of the α-chitins, and α- and β-chitins have different molecular chain packing, antiparallel, and parallel modes, respectively. Moreover, it is well-known that squid pen β-chitins have intermolecular forces weaker than those of α-chitins.[4,8–10] These differences between squid pen β-chitin and crab α-chitins may have brought about the different dispersion behavior in water at pH 3-4 by the ultrasonication treatment. However, further studies are needed to make clear the reasons for the different dispersion behavior between α- and β-chitins.

4.3.2 Characterization of squid pen β-chitin/water dispersion. Transmission electron microscopy (TEM) of squid pen β-chitin/water dispersions prepared at different pH value (pH 5, pH 4, and pH 3) was shown in Figures 4.5, 4.6, and 4.7, respectively.

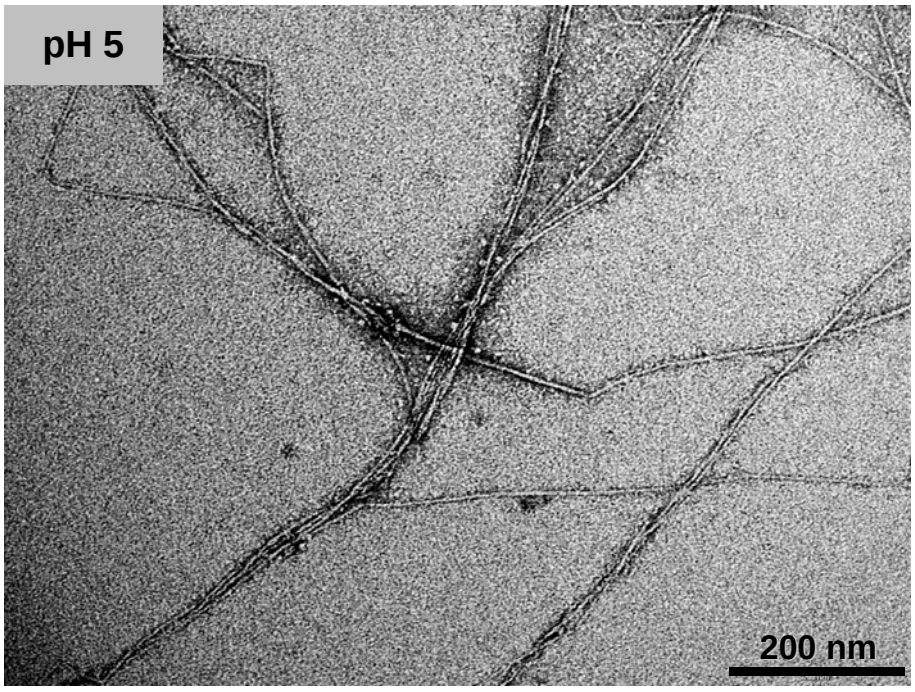

Figure 4.5. Transmission electron micrograph of chitin nano-fibers prepared from squid pen β-chitin by ultrasonication in water at pH 5 for 2 min.

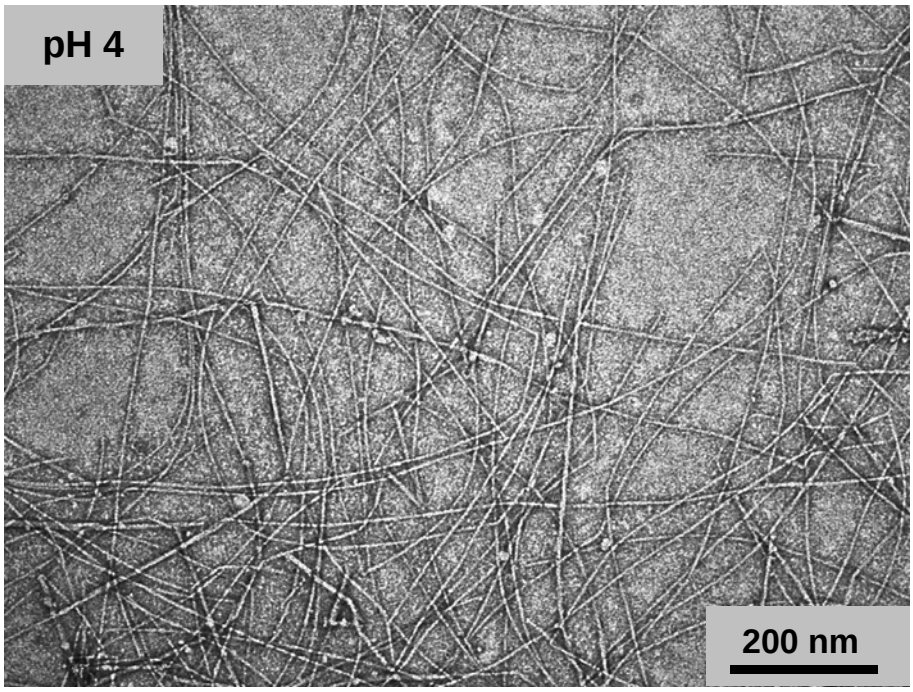

Figure 4.6. Transmission electron micrograph of chitin nanofibers prepared from squid pen β-chitin by ultrasonication in water at pH 4 for 2 min.

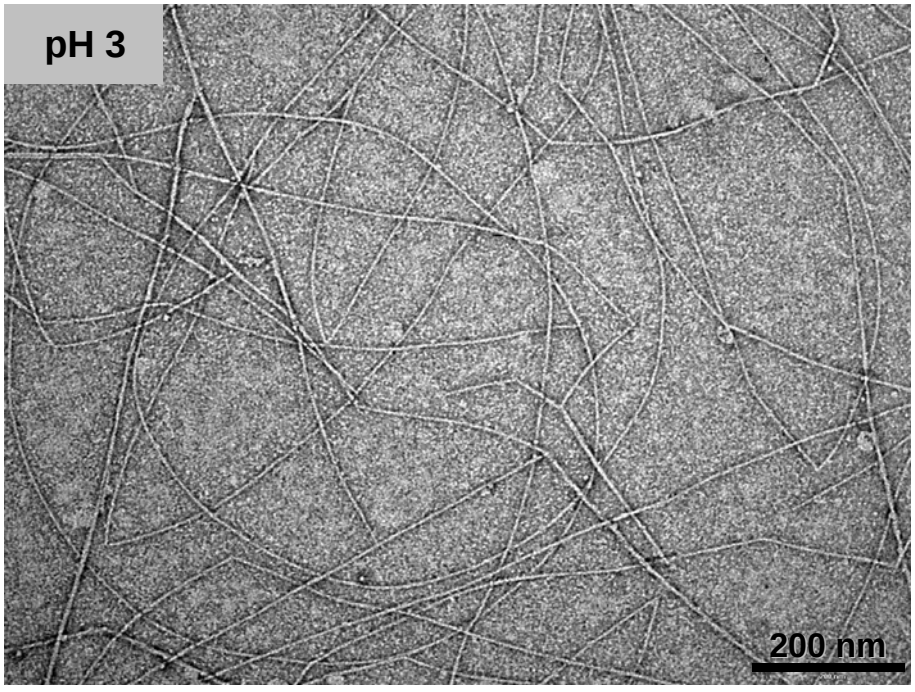

Figure 4.7. Transmission electron micrograph of chitin nanofibers prepared from squid pen β-chitin by ultrasonication in water at pH 3 for 2 min.

When squid pen β-chitins were subjected to ultrasonic treatment at pH 5, the dispersion contains lots of bundles (Figure 4.5), which results in lower light transmittance of the water dispersion (Figure 4.4). When the pH value was adjusted to 3 to 4, the TEM observations show that the dispersions consist of individualized chitin nano-fibers (Figures 4.6 and 4.7). The obtained rod-like chitin nano-fibers were evenly 3-4 nm in cross-sectional width and at least a few microns in length. Thus, nano-fibers with aspect ratios of more than 500 can be prepared from squid pen β-chitin by simple ultrasonication in water at pH 3-4.

Because the β-chitin nano-fibers observed in Figures 4.6 and 4.7 are the elements forming squid pen, complete individualization of squid pen β-chitin microfibrils can be achieved by the downsizing process, where ultrasonication is applied to the squid pen β-chitin/water slurries at pH 3-4.

ζ-Potentials of the squid pen β-chitin nano-fibers in water at pH 3-7 were then measured, and the average values are shown in Figure 4.8. The chitin nano-fibers have high cationic surface charges of more than +60 mV in water at pH 3-4, and these values decrease with increasing the pH. Thus, again the complete cationization of the C2 amino groups in the squid pen β-chitin at pH 3-4 is necessary to prepare transparent chitin nano-fiber/water dispersions in water by the ultrasonication treatment and to maintain the stable dispersion state by electrostatic repulsions between the nano-fibers with highly cationic surface charges.

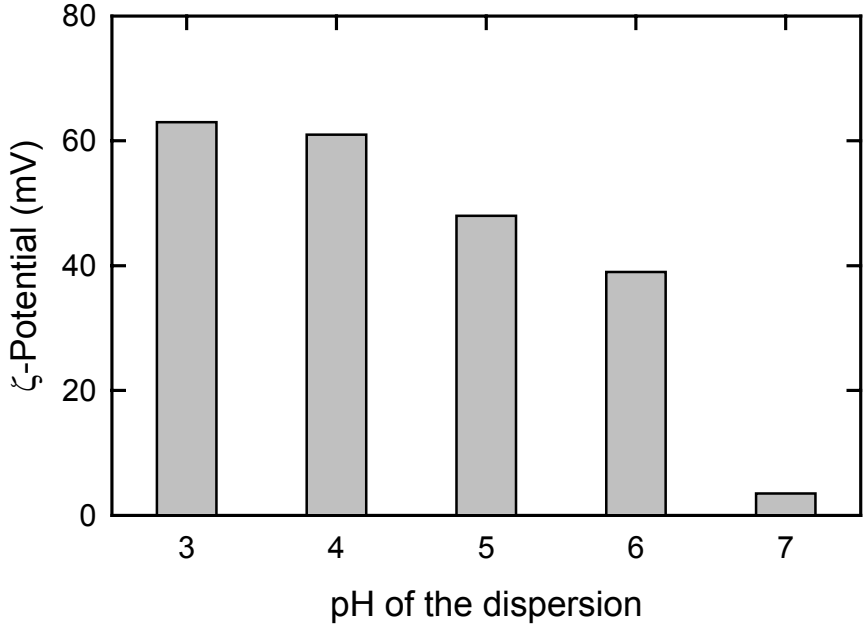

Figure 4.8. ζ-Potentials of squid pen β-chitin nano-fibers dispersed in water at pH 3-7.

Birefringence was observed at rest between cross polarizers for the transparent dispersion of squid pen β-chitin at consistency of more than 0.1% (Figure 4.9). This is because the nano-fibers have optical anisotropy and their dispersion is highly viscous even at low consistencies. Furthermore, the chitin nano-fiber/water dispersions at 0.3% consistency formed hard gels. These chitin nano-fibers, having such high aspect ratios and outstanding gel properties, are expected to be used in various fundamental and applied fields.

Figure 4.9. Squid pen β-chitin nanofibers/water dispersion at 0.2% consistency, observed at rest between cross polarizers.

4.3.3. Chemical and crystal structures of squid pen β-chitin nano-fibers. FT-IR spectra of the original squid pen β-chitin and its nano-fibers prepared by ultrasonication in water at pH 4 are shown in Figure 4.10. These two spectra are very close to each other. Because no absorption band at 1540 cm^{-1} was detected in each IR spectrum, neither the purified squid pen β-chitin nor its nano-fibers contained residual protein contaminants.[11] The degrees of N-acetylation were evaluated from the FT-IR spectra using the absorption ratios A1560/A1030, where the absorption at 1560 cm^{-1} is due to the amide II band.[11] The ratios of

A1560/A1030 for both the original squid pen β-chitin and its nano-fibers were about 0.71, which correspond to the degree of N-acetylation of about 0.9.[12] Thus, no N-deacetylation occurs on the β-chitin molecules during the ultrasonication in water at pH 4, and the chemical structure of the original squid pen β-chitin was almost unchanged by the nano-fiber conversion treatment.

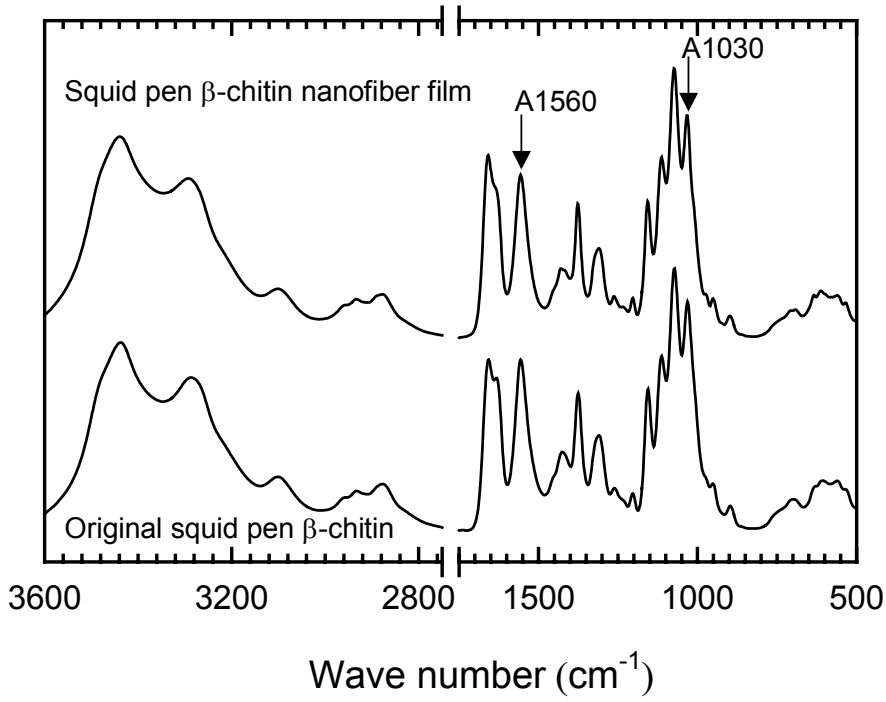

Figure 4.10. IR spectra of the original squid pen β-chitin and its nano-fibers prepared by ultrasonication in water at pH 4.

Figure 4.11 depicts X-ray diffraction patterns of the original squid pen and its nano-fibers. Both had diffraction peaks at 9.0° and 20.0°, which corresponded to the (010) plane and the (100) + (1$\bar{1}$0) + (110) mixed planes (the peak top at 20.0° was due to the (1$\bar{1}$0) plane),

respectively, of β-chitin. Thus, the original β-chitin structure remained unchanged during the process of nano-fiber preparation. Although both had typical crystal patterns of β-chitin, the crystallinity index decreased from 0.51 to 0.37 as a result of the nano-fiber conversion. The crystal sizes of the (010) plane was 4.0 and 3.5 nm for the original squid pen β-chitin and the nano-fibers, respectively, when calculated by Scherrer's equation.[13] The crystal size of the β-chitin nano-fibers, that is, 3.5 nm, was close to their cross-sectional widths (3-4 nm) measured from the TEM images (Figures 4.6 and 4.7). Both the crystal size and crystallinity of the original β-chitin were decreased slightly by the nano-fiber conversion. Probably, specific surface areas are extremely increased, and these surface areas of nano-fibers contacting water are counted as disordered regions by X-ray diffraction measurement, resulting in the partial decreases in crystallinity index and crystal size.[5]

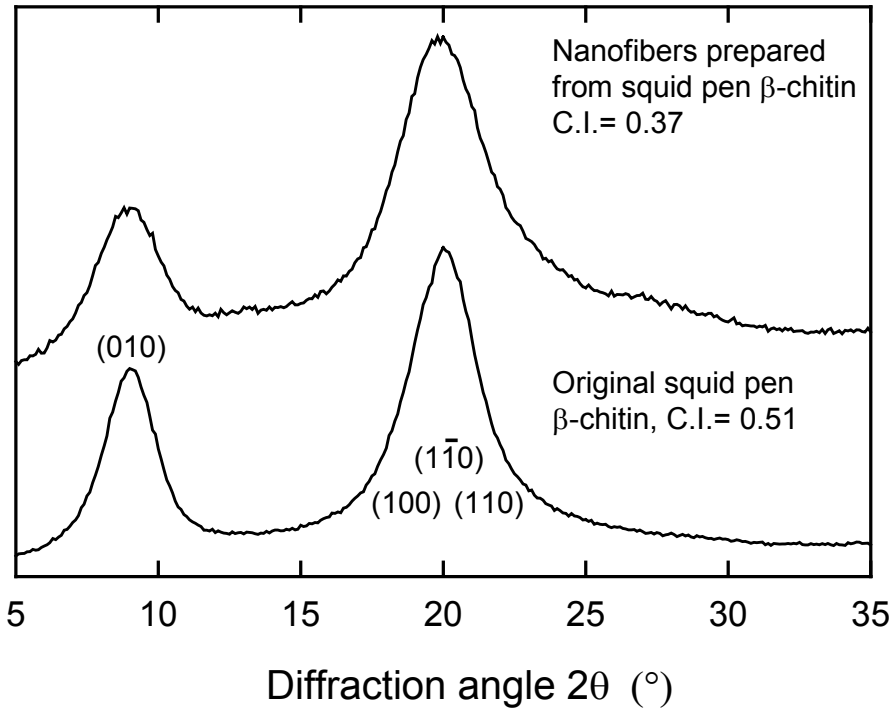

Figure 4.11. X-ray diffraction patterns and crystallinity indices (C.I.) of the original squid pen β-chitin and its nano-fibers prepared by ultrasonication in water at pH 4.

4.3.4 Squid pen β-chitin nano-fibril film. As described previously, long β-chitin nano-fibers were obtained from squid pen chitin by simple disintegration in water at pH 3-4. Figure 4.12 showed transparent films prepared from squid pen β-chitin nano-fiber/water dispersions and optical and mechanical properties of the obtained films.

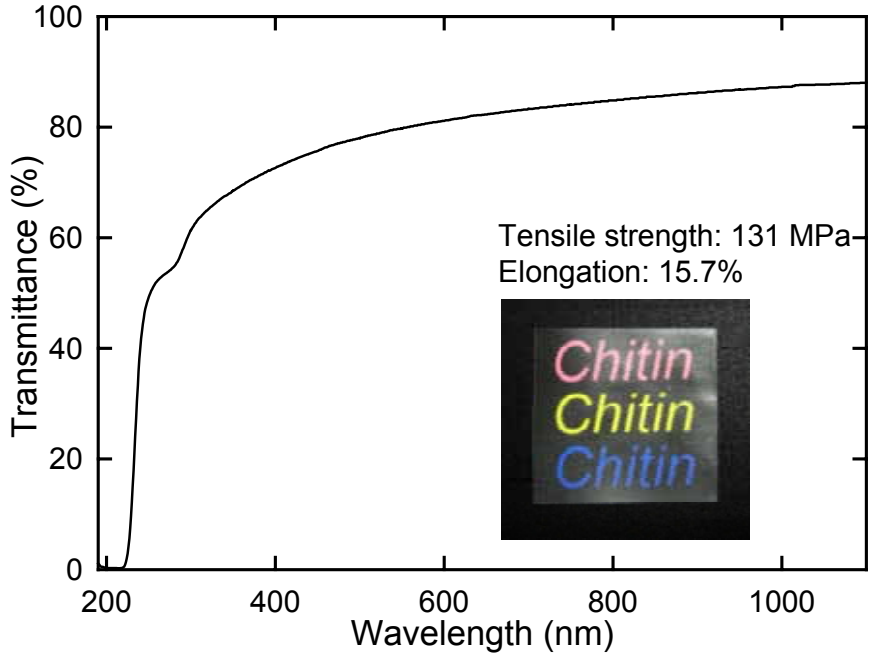

Figure 4.12. UV-vis light transmittance of squid pen β-chitin nanofiber films, the mechanical properties (tensile strength and elongation at break) with the film photo were also shown as inserts.

The films are transparent, and their UV-vis light transmittances are as high as 80-90%, resulting from sufficiently individualized squid pen β-chitin fibrils dispersed at nano-level. The tensile strengths 130 MPa of the squid pen β-chitin nano-fibril films are comparable to

those of cellophane films, but are lower than those of TEMPO-oxidized cellulose nano-fiber (TOCN) films,[6] which might be due to relatively lower crystallinity of squid pen β-chitin nano-fibers than that of TEMPO-oxidized cellulose nano-fibers.

4.3.5 Squid pen β-chitin nano-fibril aerogel. When the squid pen β-chitin nano-fiber/water dispersions were freeze-dried, soft sponge-like aerogels were obtained (Figure 4.13).

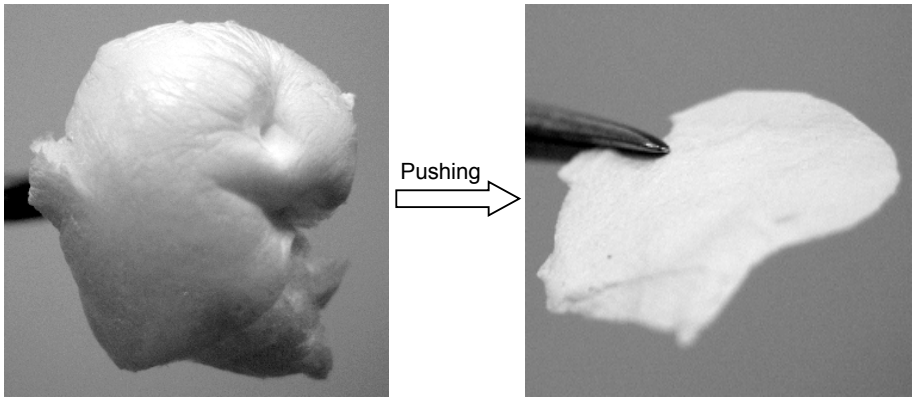

Figure 4.13. Photos of squid pen β-chitin nano-fibril aerogels prepared by freeze-drying. The left was taken just after freeze-drying, which represents the sponge-like form of the aerogel. The right was taken after pushing of the sponge-like aerogel by fingers, indicating soft properties of the areogel.

SEM images of cross-section of the squid pen β-chitin nano-fibril aerogel are shown in Figure 4.14. Porous structures of squid pen β-chitin nano-fibril aerogels were clearly observed.

The fibrils consisted of bundles and cross-linked nano-fibrils with widths around 10 nm. The widths of squid pen β-chitin nano-fibrils present in the aerogels are slightly larger than those observed for squid pen β-chitin nano-fibers dispersed in water at pH 3-4 by TEM. Thus, some aggregation might have occurred during freeze-drying treatment of the dispersions.

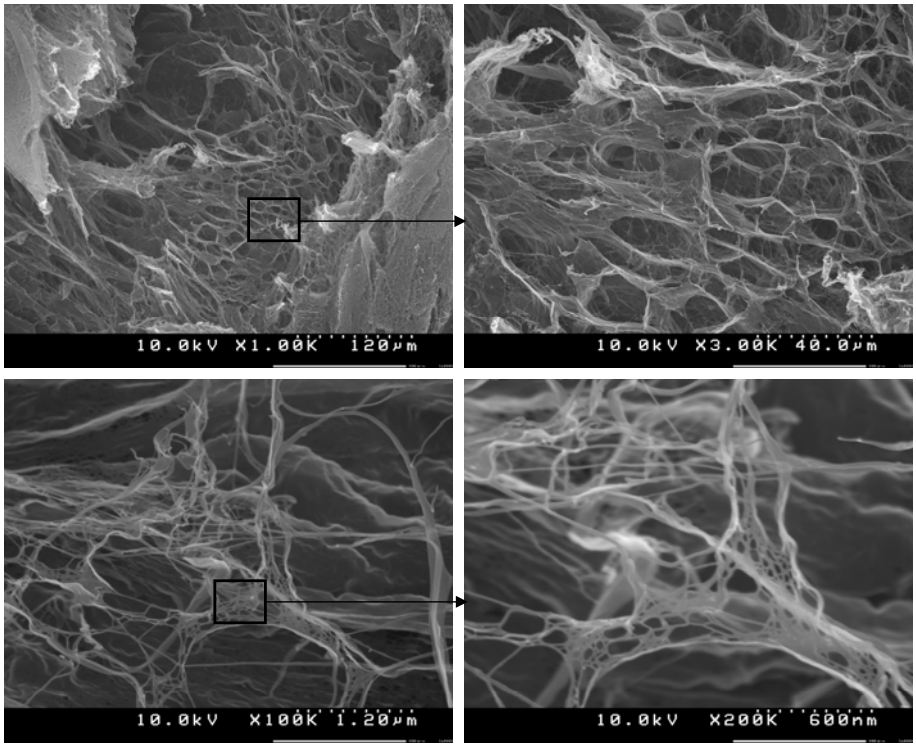

Figure 4.14. SEM images of squid pen β-chitin nano-fibril aerogels at different magnifications.

4.4 Conclusions

The work presented here highlights the procedure for preparing new individualized chitin nano-fibers of 3-4 nm in cross-sectional width and at least a few microns in length from squid pen β-chitin by simple mechanical treatment in water at pH 3-4 without any chemical modification. The original crystal structure of β-chitin was unchanged, although crystallinity index decreased from 0.51 to 0.37 as a result of the nano-fiber conversion. Protonation or cationization of the C2 amino groups present on the crystallite surfaces of the squid pen β-chitin under acid conditions is likely to be one of the most significant and necessary conditions for the nano-fiber conversion. Highly cationic ζ-potentials of the squid pen β-chitin nano-fibers in water at pH 3-4 supported the above hypothesis. Other conditions may include the relatively low crystallinity, the parallel chain packing mode, and relatively weak intermolecular forces of squid pen β-chitin, which are different from those of α-chitins. Due to the high aspect ratios of individualized squid pen β-chitin nano-fibrils (3-4 nm in width and at least a few microns in length with aspect ratios of more than 500), transparent films with high strength or sponge-like aerogels after freeze-drying were obtained.

4.5 References

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Chapter 5
Individual Chitin Nano-Whiskers Prepared from Partially Deacetylated α-Chitins by Fibril-Surface Cationization

5.1 Introduction

In the previous chapters, two new methods to prepare chitin nano-fibers/whiskers under mild conditions were developed. By TEMPO-mediated oxidation and the following mechanical treatment, α-chitin nano-whiskers and β-chitin nano-fibers were prepared from crab shell and tubeworm chitins, respectively. In the case of squid pen β-chitins, nano-fibers can be obtained by direct disintegration in water under acid conditions without any chemical modification, i.e. cationization method. Individual chitin nano-fibers 3-4 nm in cross-sectional width and at least a few microns in length were successfully prepared from squid pen β-chitin by simple mechanical disintegration in acidic water at pH 3-4. In this case, surface cationic charges are formed at the glucosamine units on the fibril surfaces, which then bring about the inter-fibrillar electrostatic repulsion in water. Thus, the opposite charges to TEMPO-oxidized cellulose or chitin nano-fibrils are formed on the surfaces of squid pen β-chitin fibrils. This simple disintegration method to prepare nano-fibrils of squid pen β-chitin has an advantageous point in terms of safety issues in application to functional foods, cosmetics and related fields, because the protocol consists of no chemical modification. However, this individualization of fibrils was not available to α-chitins under any conditions tested; only squid pen β-chitin can be converted to long and individual fibrils by the simple method probably because of its low crystallinity and some structural specifities.

α-Chitins are more abundantly present in nature originating from shells of crab, shrimp etc., and purified α-chitins are commercially available, compared with squid pen β-chitin. Hence, α-chitins rather than β-chitins are preferable resources for individualization to nano-fibrils. Grinding of α-chitin in water at pH 3 provided fibril bundles 10–20 nm in width.[1]

Based on X-ray diffraction patterns of α-chitins, widths of individual fibrils should be around 6 nm. Efforts are, therefore, still needed to obtain completely individualized α-chitin nano-fibrils in high yields without any additional chemical modifications for safety issues.

In this study, partial deacetylation was applied to a commercial α-chitin to increase the C2-primay amino groups selectively on the crystalline fibril surfaces. If cationic charge densities on crystalline fibril surfaces of α-chitin can be raised, individualization may be achieved by enhanced electrostatic repulsion between cationically-charged fibrils in high densities through disintegration in water under acidic conditions. When 50% NaOH at 90°C was applied to α-chitin, partial deacetylation occurred also inside crystallites, resulting in some decreases in crystallinity.[2] Thus, other conditions are needed to be sought for partial and position-selective deacetylation of α-chitin crystallite surfaces. Of course, formation of water-soluble deacetylated chitins or chitosans under acidic conditions should be avoided as much as possible.[3]

5.2 Materials and Methods

5.2.1 Materials. Purified α-chitin powder originating from crab shell was a commercial product (TCI Laboratory Chemicals Co., Japan). Sodium hydroxide, sodium borohydride and other chemicals and solvents were of laboratory grade (Wako Pure Chemicals Co., Japan), and used as received.

5.2.2 Partial deacetylation. The α-chitin (1 g) was suspended in 33% (w/w) NaOH (25 ml) containing 0.03 g $NaBH_4$ to prevent alkali-provoked depolymerization and weight loss, and the slurry was heated at 90°C for 1-4 h with occasional shaking every 15-20 min. The partially deacetylated chitin was collected, and washed thoroughly with de-ionized water by repeated centrifugation at 4100 g for 15 min to neutrality. A part of the wet NaOH-treated product was freeze-dried for further analyses, and the rest was kept in wet state at 4°C. The deacetylation of α-chitin was also carried out at room temperature for 12-48 h according to the same procedure as above described.

5.2.3 Determination of degree of *N*-acetylation (DNAc). DNAc values of the original and NaOH-treated chitins were calculated from both cationic charges and nitrogen/carbon contents determined by electric conductivity titration method and elementary analysis using a Thermo Finnigan Flash EA1112, respectively.[4-6] In the former case, to a dried sample (0.1 g) were added water (60 ml) and a small amount of 0.5 M NaOH to adjust the pH to 9. The mixture was stirred for 30 min to prepare a well-dispersed slurry. Then, 0.1 M HCl was added to the mixture to set the pH in the range of 2.5-3.0. A 0.05 M NaOH solution was added at a rate of 0.1 mL/min up to pH 11 using a pH-stat titration system. The conductivity and pH curves obtained reflected contents of C2-amino groups in the chitins.[4]

5.2.4 X-ray diffraction analysis. The original or partially deacetylated chitin (0.1 g) was converted to a pellet 1 cm in diameter by pressing at ca. 750 MPa for 1 min using a disc apparatus, and subjected to X-ray diffraction analysis. Details were described in page 13, "2.2.4 X-ray diffraction analysis", in chapter 2.

5.2.5 FT-IR spectroscopy. FT-IR spectra of the original and partially deacetylated chitins were recorded with 4 cm^{-1} resolution and 64 scans on a Nicolet Magna 860 (Madison, WI, USA) in the transmission mode. All of the chitin samples were converted to KBr disks for FT-IR analysis. Details were described in page 13, "2.2.7 FT-IR spectroscopy", in chapter 2.

5.2.6 Mechanical disintegration. The partially deacetylated chitins in wet state were suspended in water at 0.1% (w/v) consistency. The original pHs of the slurries were 6-7, and the pHs of some slurries were adjusted to 3-4 by adding several drops of acetic acid to the slurry. Each slurry was agitated using a magnetic stirring bar at 1200 rpm for 5 days, and finally subjected to sonication for 1 min using an ultrasonic generator (US-300T, Nihoseiki Co., Japan).

5.2.7 Analyses of the dispersions. The above chitin dispersions at 0.1% consistency were introduced into a poly(methyl methacrylate) disposable cuvettes, and the transmittances

were measured from 300 to 750 nm wavelength using a Shimadzu UV-Vis spectrophotometer (UV-1700). Details were described in page 55, "4.2.3 Optical transmittance", in chapter 4. The transmission electron micrographs (TEM) observation was carried out according to the procedure described in page 14, "2.2.8 Microscopic observations", in chapter 2; All of the TEM images were collected with a charge coupled device (CCD) camera and recorded using iTEM® software. A Thermo HAAKE rotational rheometer (Rheo-Stress 600) was used to examine the flow properties of 0.1% chitin nano-whisker/water dispersions. The dispersion (410 μL) was transferred to a titan cone-plate sensor system with a 2° cone angle and a 35 mm diameter. Measurements were carried out at 24°C for 3 min. Flow curves were recorded by HAAKE rheometer software (RheoWin version 3.14). Viscosity of 0.1% chitin nano-whisker or nano-fibril/water dispersions was calculated from shear stress at 114 s^{-1} using the Newtonian equation for viscosity: viscosity (η) = shear stress (τ)/shear rate ($d\gamma/dt$). The TEMPO-oxidized α-chitin dispersion, 3 M HCl-hydrolyzed α-chitin dispersion and a chitosan 1000 solution were prepared and used as references in some analyses, the preparation procedure were as following. 1) The α-chitin was oxidized by TEMPO-mediated oxidation system using 5.0 mmol/g of NaClO as co-oxidant followed by ultrasonic treatment in water. The details are shown in chapter 2; 2) HCl-hydrolyzed α-chitin dispersion was prepared according to the reported method by using 3 M HCl;[4] 3) Chitosan 1000 solution was prepared by dissolution of chitosan 1000 (Wako Pure Chemicals Co., Japan) in acidic water at pH 3-4.

5.3 Results and Discussion

5.3.1 Partial deacetylation of α-chitin at 90°C. Figure 5.1 shows the relationship between the treating time of α-chitin with 33% NaOH at 90°C and the degree of N-acetylation (DNAc) of the products. The DNAc was decreased from 0.90 to 0.77 by the NaOH treatment for 1 h and then gradually decreased to 0.70 for 4 h, when determined by conductivity titration method. The DNAc determined by the elementary analysis was similar to that determined by the conductivity titration at each point, although some discrepancies in DNAc values were observed between the two methods. The conductivity titration might have preferably detected primary amino groups present on outer surfaces of α-chitin fibrils.[2] The

C2-primary amino group contents, which were calculated from the DNAc values obtained by the conductivity titration, are also plotted in Figure 5.1. The C2-NH_2 content increased from 0.48 mmol/g to 1.56 mmol/g by the NaOH treatment for 4 h. Hence, when the C2-NH_2 groups are protonated under acid conditions, significant amounts of cationic charges are possible to be formed in the products. These results show that partial deacetylation of α-chitin can be achieved by the 33% NaOH treatment under the conditions used. Even though small amounts of the products were lost by handling during the repeated centrifugation treatments, high solid recovery ratios of 85–90% were maintained for all the products obtained.

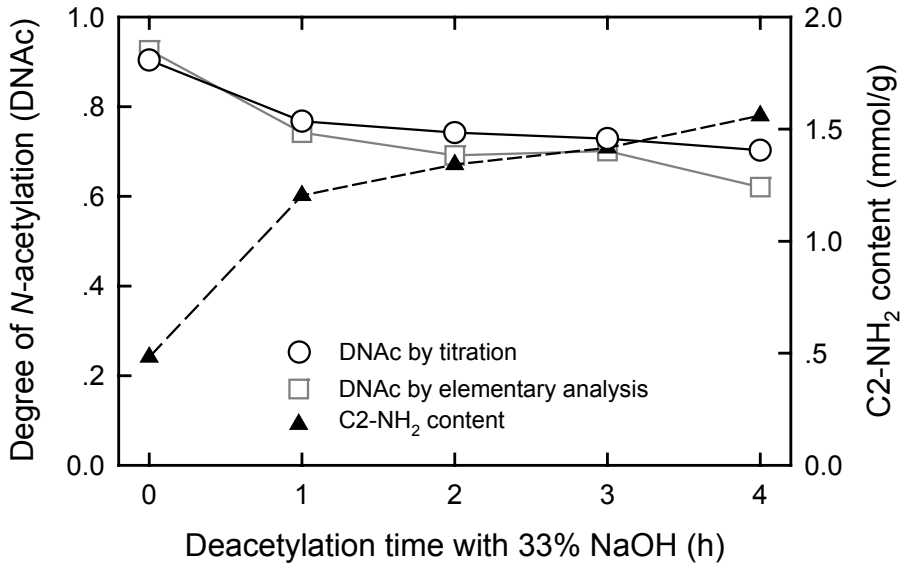

Figure 5.1. Relationship between 33% NaOH treating time of α-chitin at 90°C and either degree of N-acetylation (DNAc) or C2-amino group content in the products. DNAc values were determined by conductivity titration method and elementary analysis, and the C2-NH_2 contents were calculated from the DNAc values determined by the conductivity titration.

Chemical structures of original and partially deacetylaed α-chitins were determined by FT-IR spectra, as shown in Figure 5.2. The spectra of original and partially deacetylated α-chitins are very close to each other. The degree of N-acetylation (DNAc) can be evaluated from the FT-IR spectra using the absorption ratio A1560/A1030 and the baseline in Figure 5.2, where the absorption at 1560 cm^{-1} and 1030 cm^{-1} is due to amide II and the C-O stretching vibration of the chitin skeleton band respectively.[7,8] The ratio decreased from original 0.76 to 0.66-0.63 when α-chitins were treated by 33% NaOH at 90℃ for 2-4 h, which is corresponding to the degree of N-acetylation (DNAc) *ca*.0.70. The value was similar to the results obtained by either conductivity titration method or elementary analysis (Figure 5.1).

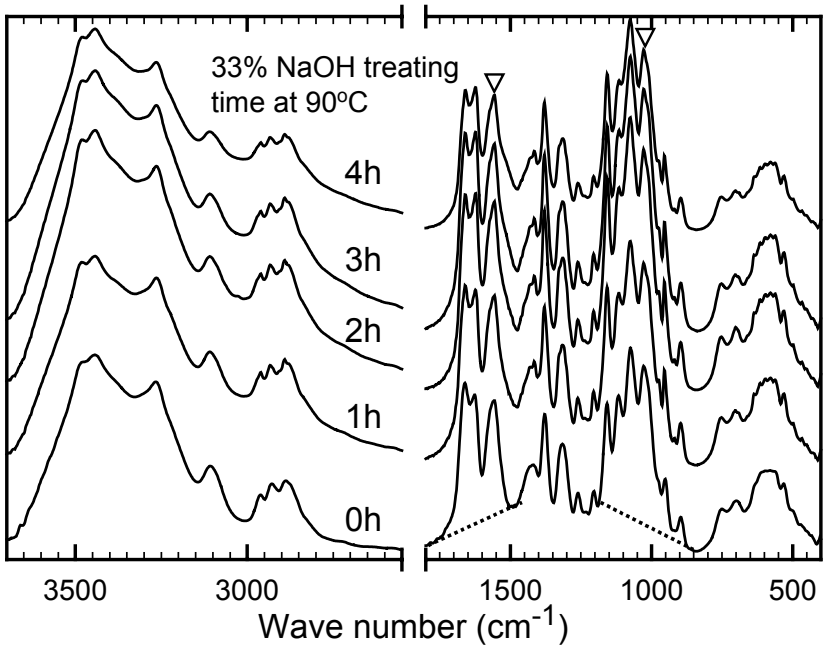

Figure 5.2. IR spectra of the original and partially deacetylated chitins prepared by 33% NaOH treatment at 90°C for 1-4 h.

Crystal structures of the 33% NaOH-treated products were investigated from their X-ray diffraction patterns (Figure 5.3). All the partially deacetylated chitins had typical diffraction patterns of α-chitin; the original α-chitin structure was maintained during the NaOH treatment up to 4 h. The crystal sizes of the (110) plane and crystallinity values were calculated from the X-ray diffraction patterns (Figure 5.4). The crystal size 6.7 nm and high crystallinity 95% of the original α-chitin were slightly decreased to 6.4 nm and 92%, respectively, by the 33% NaOH treatment for 4 h. These results revealed that the partial deacetylation with 33% NaOH at 90°C for 1-4 h almost selectively takes place on the surfaces of α-chitin crystallites.

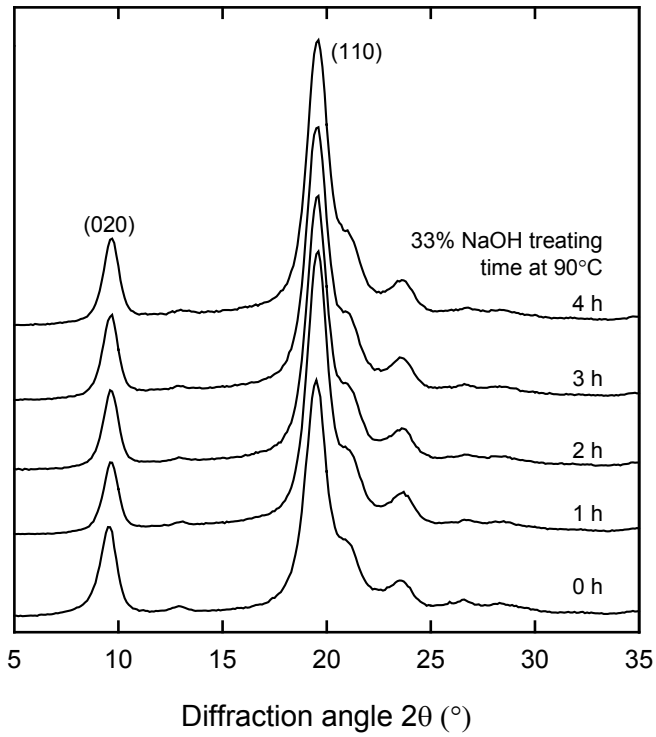

Figure 5.3. X-ray diffraction patterns of the original and partially deacetylated chitins prepared by 33% NaOH treatment at 90°C for 1-4 h.

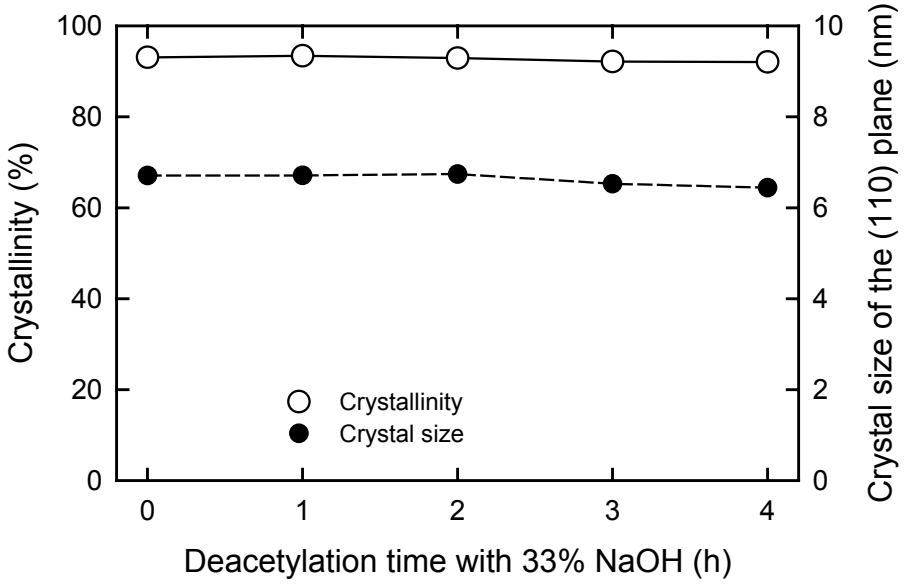

Figure 5.4. Crystallinity and crystal size of the (110) plane of partially deacetylated chitins prepared by 33% NaOH treatment at 90°C for 1-4 h.

When 40% NaOH was used in preliminary experiments, significant decrystallization was observed for the products, indicating that partial deacetylation occurred also inside α-chitin crystallites. On the other hand, it took longer time than 12 h for obtaining partially deacetylated chitins with the same DNAc values, when 20% NaOH at 90°C was adopted. Thus, the 33% NaOH treatment was suitable for partial and position-selective deacetylation of α-chitin crystallite surfaces.

5.3.2 Partial deacetylation of α-chitin at room temperature. Figure 5.5 showed IR spectra of the original and partially deacetylated chitins prepared by 33% NaOH treatment at room temperature for 12-48 h. The results were quite similar to the partially deaectylated α-

chitins prepared by 33% NaOH treatment at 90°C for 1-4 h. The spectra of original and partially deacetylated α-chitins are close to each other, and the absorption ratio A1560/A1030 decreased from original 0.76 to 0.64 when α-chitins were treated by 33% NaOH at room temperature for 48 h. Compared with the deacetylation occurred at 90°C, where the absorption ratio A1560/A1030 decreased to 0.63 when α-chitins were treated for 4 h, longer time more than 24 h is needed to achieve the similar degree of N-acetylation (DNAc) at room temperature.

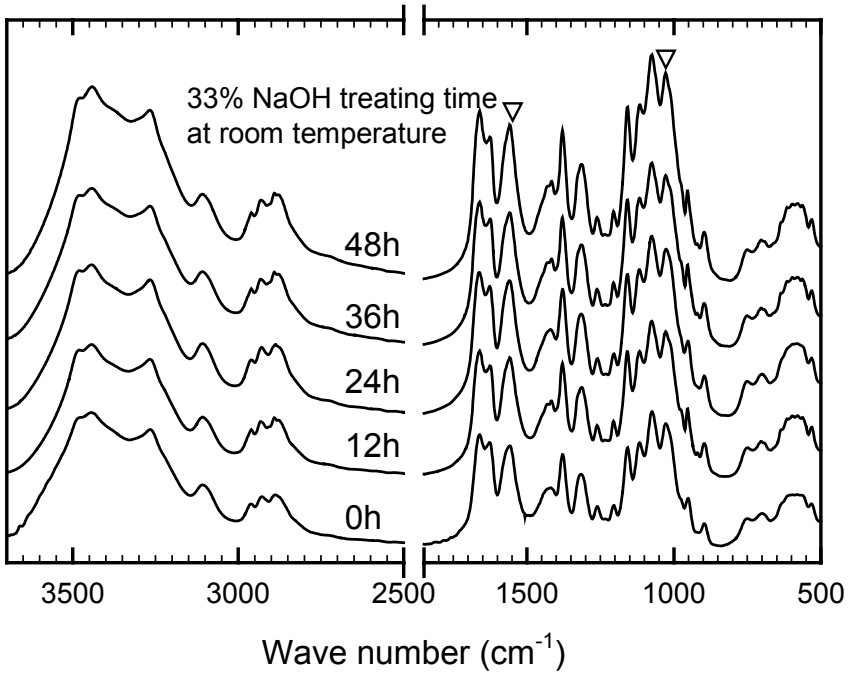

Figure 5.5. IR spectra of the original and partially deacetylated chitins prepared by 33% NaOH treatment at room temperature for 12-48 h.

The X-ray diffraction analysis of partially deacetylated chitins prepared at room temperature also showed similar patterns to the original α-chitins and the partially deacetylated chitins prepared at 90°C (Figure 5.6). The original α-chitin structure was maintained during the NaOH treatment up to 48 h at room temperature, even though the crystal sizes of the (110) plane and crystallinity values were slightly decreased to 6.68 nm and 90%, respectively (Figure 5.7).

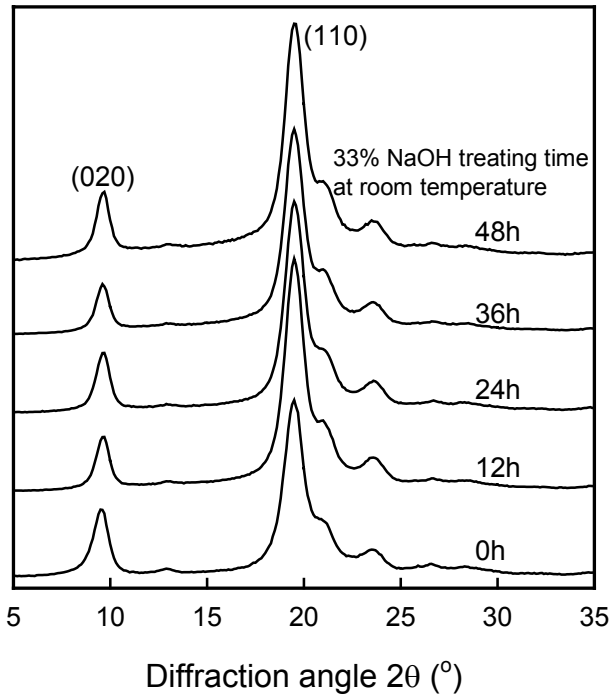

Figure 5.6. X-ray diffraction patterns of the original and partially deacetylated chitins prepared by 33% NaOH treatment at room temperature for 12-48 h.

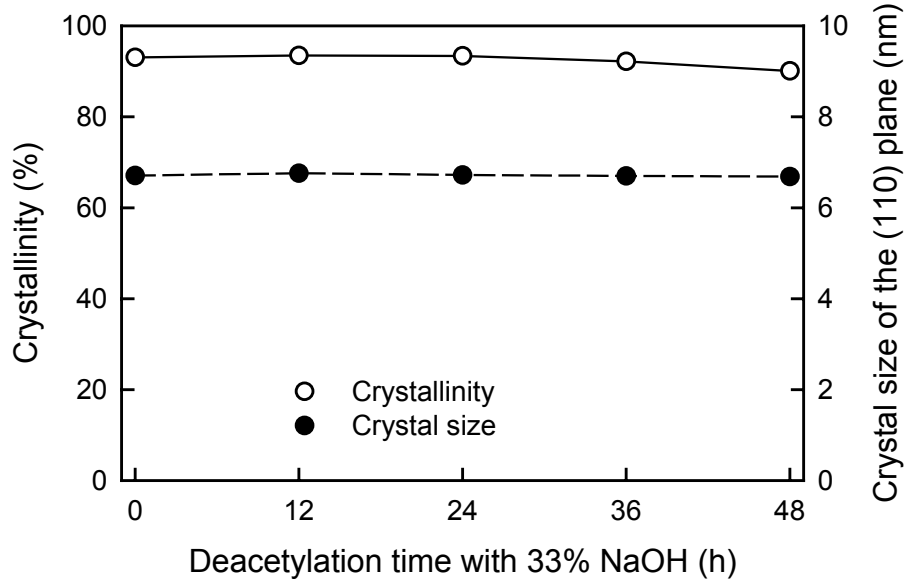

Figure 5.7. Crystallinity and crystal size of the (110) plane of partially deacetylated chitins prepared by 33% NaOH treatment at room temperature for 12-48 h.

Hence, the partial deacetylation with 33% NaOH can also be carried out at room temperature, similar to the NaOH treatment at 90°C for 1-4 h, the deacetylation almost selectively takes place on the surfaces of α-chitin crystallites. However, longer time is needed to achieve desired degree of N-acetylaiton at room temperature.

5.3.3 Disintegration of partially deacetylated chitins in water. When the partially deacetylated chitins with DNAc of 0.74-0.70 prepared either at 90°C or room temperature were stirred in water at pH 3-4, they were finally turned to highly viscous and transparent gels (Figure 5.8 and Figure 5.9). Solution-state ^{1}H- and ^{13}C-NMR spectra of the gels revealed that nearly no deacetylated α-chitin molecules were present as a soluble fraction at molecular level

in the gels. Thus, the transparent gels probably consisted of solid fibrils of partially deacetylated α-chitin dispersed at nano level in water at pH 3-4. On the other hand, when the partially deacetylated chitins were disintegrated in water at pH 6-7, the slurries were not turned to transparent gels; cationization or protonation of the C2-NH_2 groups at pH 3-4 is required for conversion of the partially deacetylated chitins to transparent gels by mechanical disintegration in water. The original α-chitin with DNAc of 0.90 was slightly swollen in water at pH 3-4 even after extended stirring time or much harsh disintegration treatments using an ultrasonic and mechanical homogenizers. However, no transparent gels could be obtained[3], because the cationic charge density of the fibril surfaces was insufficient in this case.

Birefringence was observed at rest between cross polarizers for the transparent dispersions of the partially deacetylated α-chitins at consistency of more than 0.1%. This optical anisotropy indicates that the transparent gels with high viscosities even at low solid contents consist of chitin fibrils dispersed at nano level.

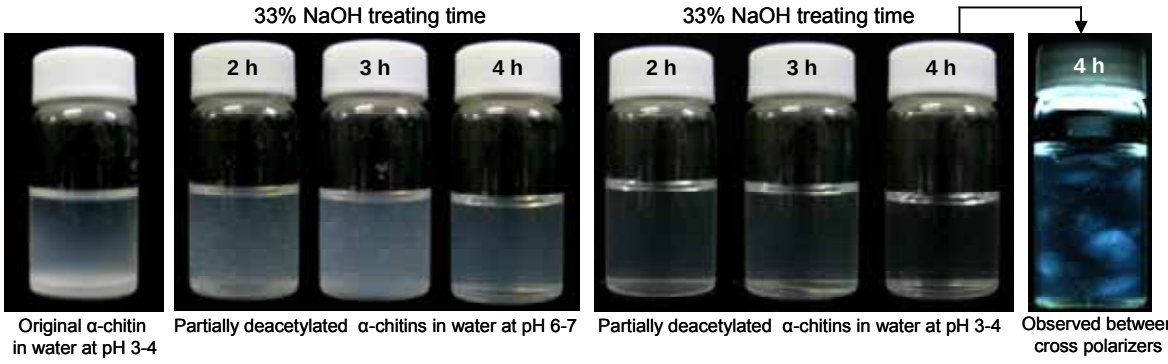

Figure 5.8. Dispersion states of the original and partially deacetylated α-chitins prepared at 90°C in water at different pHs.

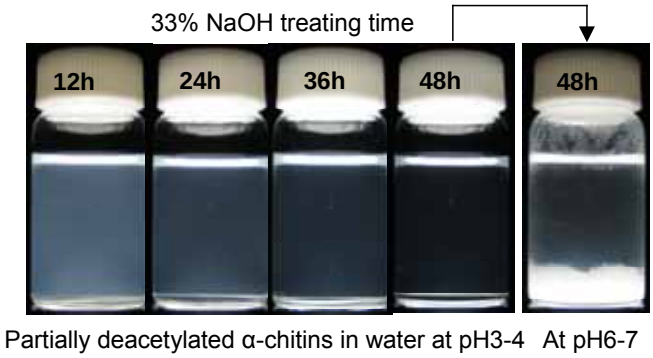

Figure 5.9. Dispersion states of the partially deacetylated α-chitins prepared at room temperature in water at different pHs.

In this experiment, we adopted the agitation of slurries using a magnetic stirrer bars for gentle mechanical treatment for 5 days and the successive ultrasonication for 1 min to obtain transparent gels. When the combination of ultrasonic and double-cylinder-type homogenizers was used for disintegration in water at pH 3-4, similar transparent gels were obtained within 10 min. However, this harsh mechanical treatment might have brought about some damages to chitin molecules and fibrils. And, the yield of the nano-dispersed chitins obtained by such homogenizer treatments were lower than those prepared by the adopted disintegration procedure using a magnetic stirrer bar.

TEM observations revealed that the gels consisted of rod-like nano-whiskers, showing that individualization of α-chitin fibrils can be achieved by the partial deacetylation with 33% NaOH at 90°C for 3 h and the following mechanical disintegration in water at pH 3-4 (Figure 5.10). It is noteworthy that some long and individual fibrils with widths similar to those of the whiskers but lengths of approximately 1 μm were observed in the TEM images, which have been never reported so far for any crab or shrimp shell α-chitin fibrils. Position-selective partial deacetylation in combination with gentle agitation of the slurries in water at pH 3-4 might have resulted in the effective separation and individualization of α-chitin fibrils having less mechanical damages.

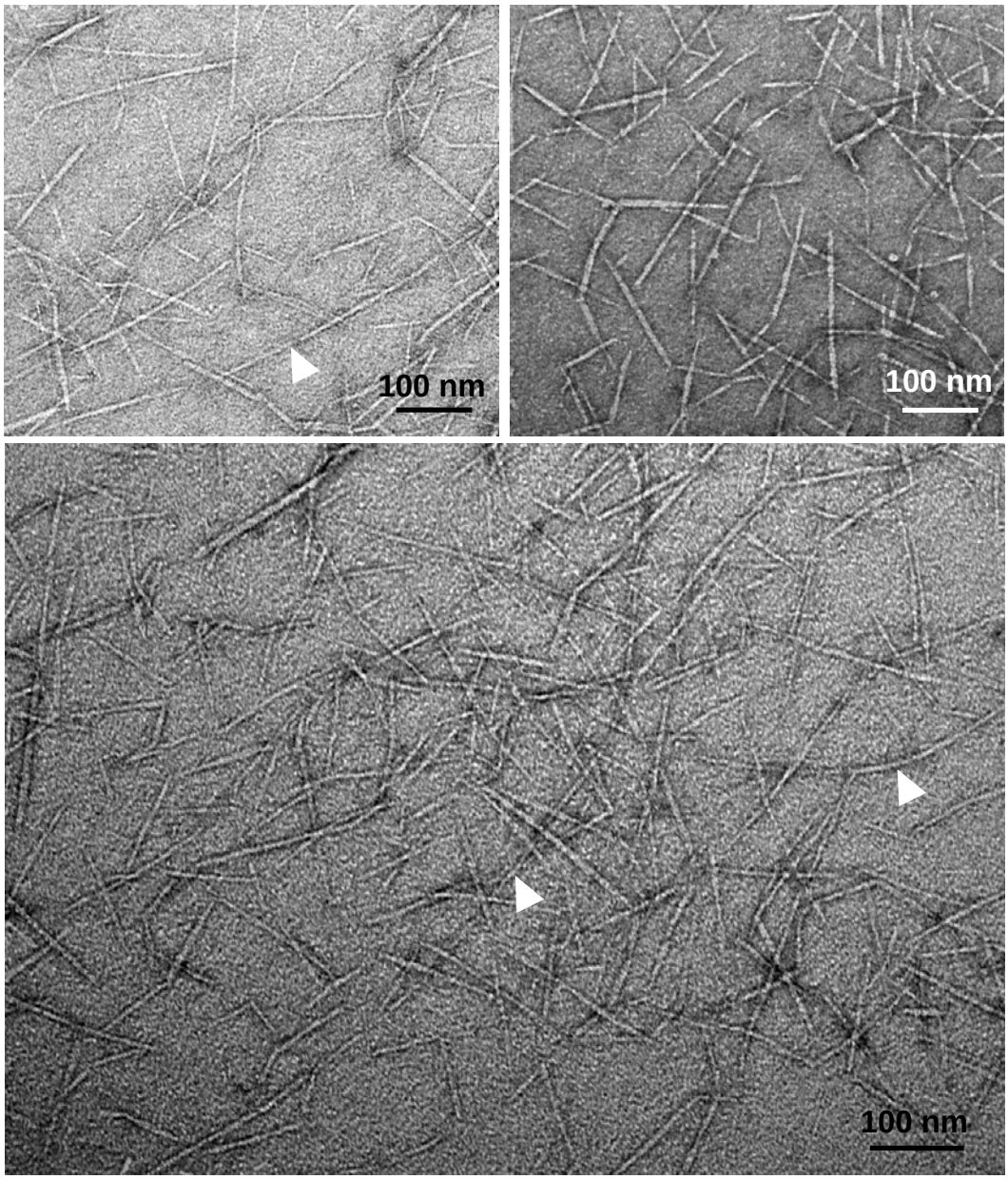

Figure 5.10. TEM images of partially deacetylated chitin with DNAc 0.73 prepared from α-chitin by 33% NaOH treatment at 90°C for 3 h followed by disintegration in water at pH 3–4. Arrows indicate the presence of long fibrils.

The whiskers had average length and cross sectional width of 250±140 nm and 6.2±1.1 nm, respectively, from approximately 100 individual whiskers in TEM images (Figure 5.11). The nano-whisker widths measured from TEM images corresponded well to the crystal size of the (110) plane determined from X-ray diffraction pattern of the same sample (Figure 5.4 and Figure 5.7). Although the widths had narrow distribution, the length distributed widely and randomly. The individualization of the partially deacetylated α-chitin fibrils in water at pH 3-4 as nano-whiskers or nano-fibrils can be achieved by the following two factors. Sufficient amounts of protonated surface C2-NH_2 groups of the partially deacetylated chitin fibrils in water at pH 3-4, which brings about electrostatic repulsion between cationically charged α-chitin fibrils. Another is mechanical disintegration in water, which is associated with partial chain scission occurring at random locations along the fibrils.

Because the nano-whiskers obtained had various and random lengths, it is not plausible that the mechanical scission of the fibrils took place at some mechanically weak regions periodically present along the longitudinal direction of each α-chitin fibril. It is rather likely that each fibril undergo strong mechanical stress randomly along the longitudinal direction of each fibril during disintegration in water, resulting in the wide and random distribution of the nano-whisker lengths.

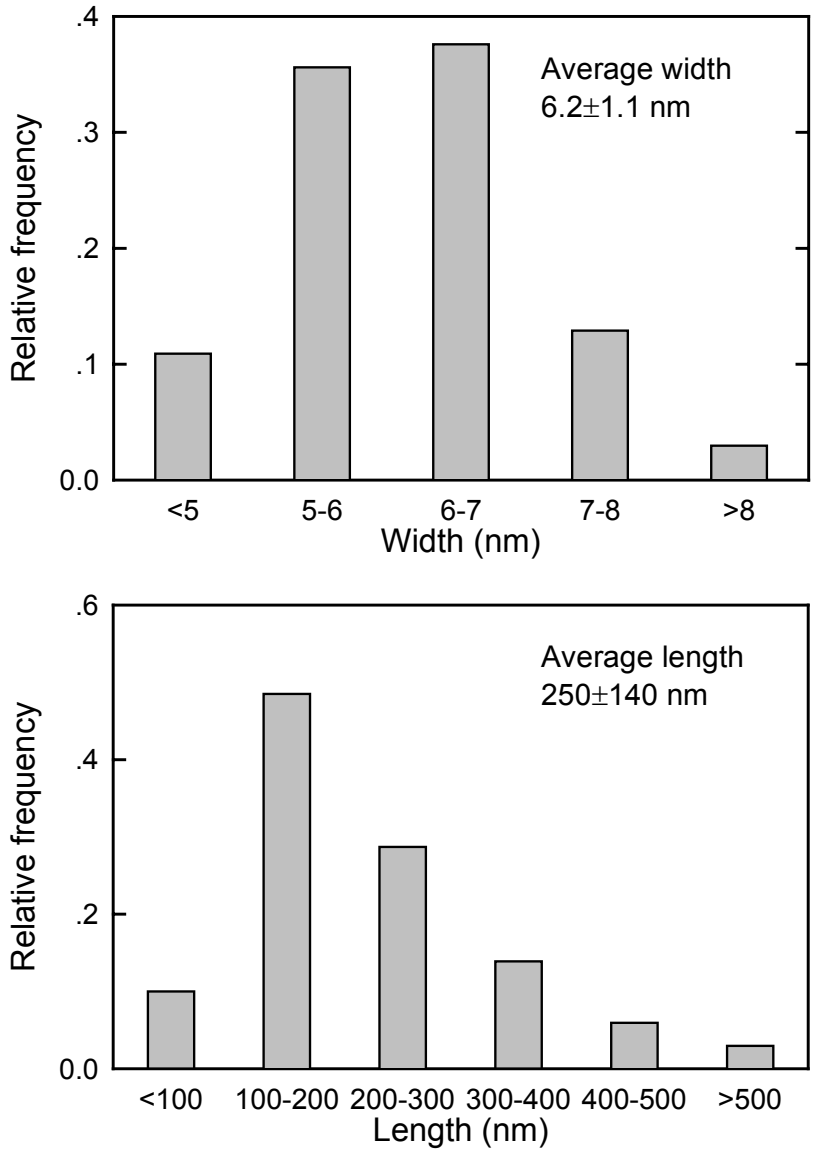

Figure 5.11. Distribution of width and length of partially deacetylated α-chitin nano-whiskers prepared by 33% NaOH treatment for 3 h and the following disintegration in water at pH 3-4. Measured from TEM images.

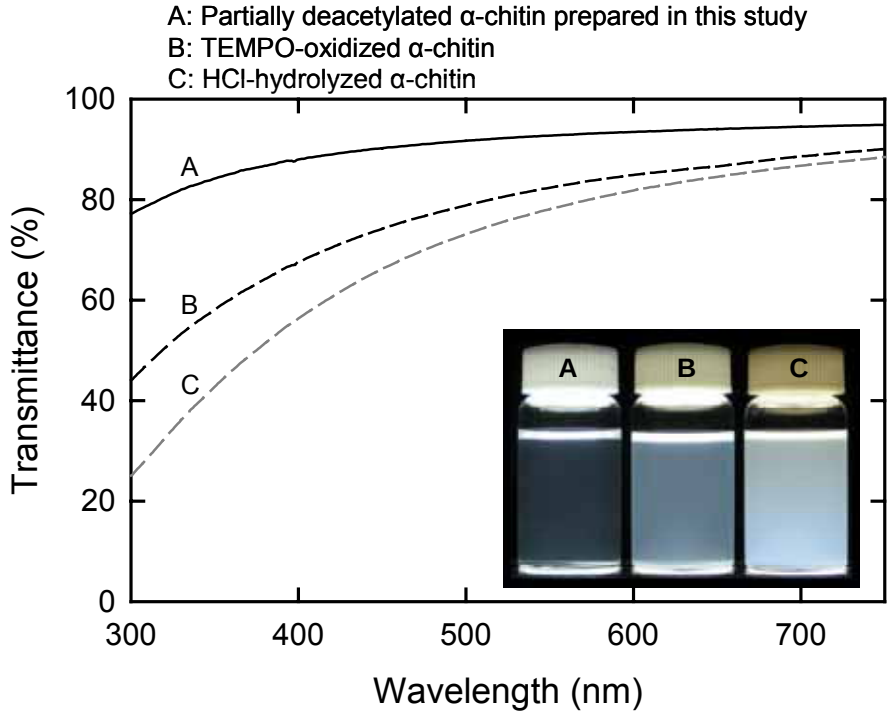

Figure 5.12. UV-visible light transmittance of 0.1% dispersions at pH 3-4 of partially deacetylated α-chitin with DNAc 0.73. TEMPO-oxidized α-chitin (chapter 2) and 3 M HCl-hydrolyzed α-chitin[4,9] dispersions at the same consistencies are also depicted as reference.

The UV-vis light transmittance of the 0.1% dispersion of the partially deacetylated chitin was depicted in Figure 5.12, showing that the highly transparent gel consisting of α-chitin nano-whiskers was obtained. The light transmittance at wavelength 500 nm was as high as 92%, whereas acid hydrolyzed α-chitin[4,9] and TEMPO-oxidized α-chitin (chapter 2) had light transmittance of lower than 80%. Thus, the individualization of α-chitin nano-whiskers in the dispersion gels developed in this study can be regarded as the highest level; introduction of cationic charges in high densities on the crystalline fibril surfaces might have

brought about such transparent gels consisting of mostly individualized α-chitin nano-whiskers. The high light transmittance was maintained without any changes for at least one month after the dispersion preparation; the partially deacetylated chitin nano-whiskers can keep the stable dispersion state without any peeling-off of the partially deacetylated chitin molecules from the surface of the crystallites in water at pH 3-4 at least for one month.

Flow properties of α-chitin nano-whisker/water dispersions prepared by different methods were also studied in this section. Relationships between shear rate of the dispersions and either shear stress or viscosity values were shown in Figure 5.13 together with those for typical chitosan solution. The TEMPO-oxidized and HCl-hydrolyzed chitin dispersions had similar flow properties, whereas the partially deacetylated chitin dispersion had similar but higher viscosity than the former two samples. All α-chitin nano-whisker/water dispersions had clearly different flow properties from those of the chitosan dissolution.

Especially, all of the α-chitin nano-whisker dispersions exhibited pseudo-plastic flows, and clearly showed yield stress in the flat region at lower shear rates, which is often observed for gel-like dispersions.[6] Such gel-like properties with yield stress behavior could not be observed for the chitosan dissolution, in which chitosan molecules were dissolved at molecular level in acidic water. Therefore, the specific flow properties of chitin nano-whisker dispersions were characteristic, different from the chitosan solution. Interestingly, even though the TEMPO-oxidized chitin nano-whiskers and HCl-hydrolyzed chitin nano-whiskers have opposite surface charges of anionic or cationic on the chitin crystallite surfaces, they showed quite similar flow properties.

From the relationships between shear rate and viscosity (Figure 5.13), shear-thinning properties were observed for all α-chitin nano-whisker dispersions even at higher shear rates, which are also different from that of the chitosan dissolution, indicating again the specific flow properties of α-chitin nano-whisker dispersions. Moreover, the partially deacetylated chitin nano-whisker/water dispersion showed obviously higher viscosity than that of the TEMPO-oxidized chitin nano-whisker/water or HCl-hydrolyzed α-chitin nano-whisker/water dispersion. The higher viscosity might be due to individualization of partially deacetylated α-chitin fibrils at higher level in acidic water, which is also explainable for higher light transmittance of the dispersion (Figure 5.12).

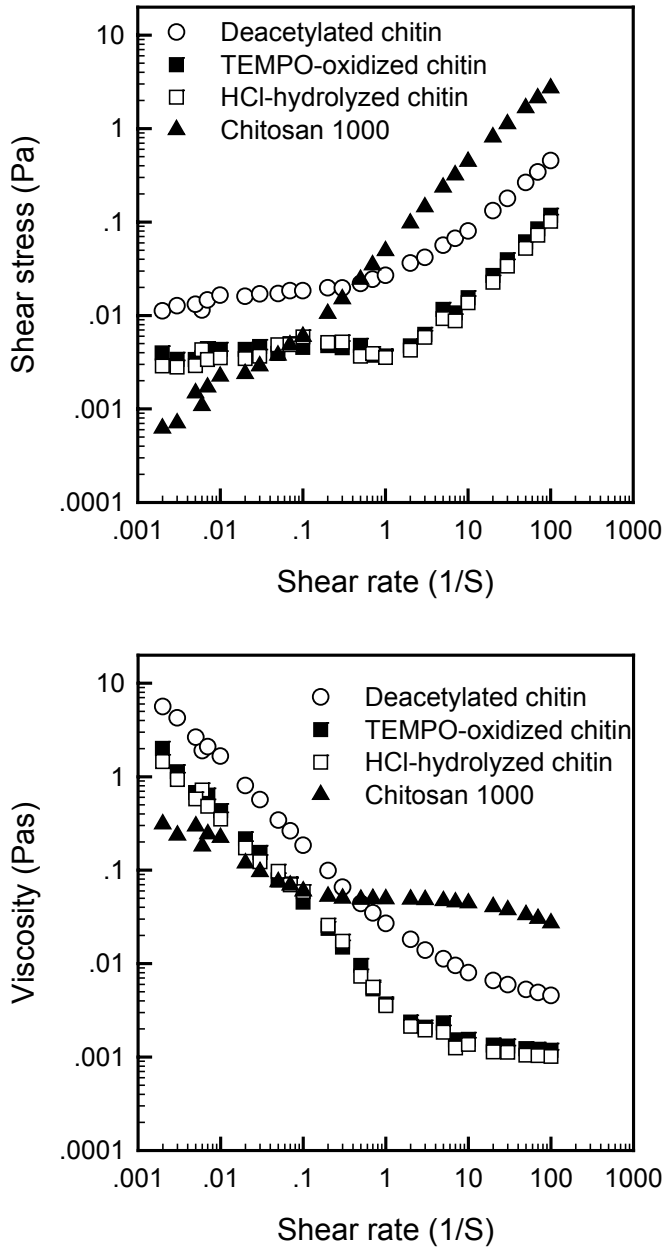

Figure 5.13. Relationships between shear rate and either shear stress (upper) or viscosity (lower) of 0.1% dispersions at pH 3-4 of partially deacetylated α-chitin with DNAc 0.73. TEMPO-oxidized α-chitin dispersion (chapter 2), 3 M HCl-hydrolyzed α-chitin dispersion,[4,9] and a chitosan 1000 solution at the same consistencies are also plotted as references.

In conclusion, the nano-whiskers of α-chitins showed clearly different flow properties from the chitosan dissolution due to some nano-dispersion effects. The partially deacetylated α-chitin nano-whiskers had the highest light transmittance and viscosity, because the smallest whisker widths were obtained by individualization of fibrils at the highest level.

Some preparation methods of α-chitin nano-whiskers and β-chitin nano-fibrils and their characteristics have been already studied. Representative procedures are: 1) hydrolysis of α-chitin with 3 M HCl and the successive homogenizer treatment for 30-45 min in water,[9] 2) deacetylation of α-chitin with 50% NaOH followed by acid hydrolysis with 3 M HCl,[2] 3) TEMPO-mediated oxidation of α-chitin under suitable conditions and the successive disintegration in water (chapter 2), and 4) simple disintegration of squid pen β-chitin in water at pH 3-4 (chapter 4). The advantageous and characteristic points of the newly developed α-chitin nano-whiskers in this chapter are as follows; 1) commercially available and pure (crab and shrimp shell-originating) α-chitins can be used as the starting materials, 2) nano-whiskers are obtained in high yields 85-90%, 3) rod-like morphology of the nano-whiskers supports the high yields, because other α-chitin nano-whiskers prepared by acid hydrolysis or TEMPO-mediated oxidation have spindle-like morphologies with larger distribution range of widths,[2,4,9-13] 4) the α-chitin nano-whisker dispersion gels developed in this chapter have high light transmittance or high transparency, indicating that individualization of α-chitin fibrils at high level can be achieved, and 5) because α-chitin nano-whiskers obtained in this chapter have a lower hurdle in terms of safety issues, compared with chemically modified new materials such as TEMPO-oxidized α-chitins, potential applications can be expanded to not only functional materials but also functional foods, life science and medical fields.

5.4 Conclusions

Mostly individualized α-chitin nano-whiskers were obtained in high yields 85-90% by partial deacetylation with 33% NaOH at 90°C for 2-4 h or at room temperature for 24-48 h and the successive disintegration in water at pH 3-4. The DNAc values of the products were 0.74-0.70, which corresponded to the C2-NH_2 contents of 1.34-1.56 mmol/g. X-ray diffraction analysis showed that crystallinity and crystal size of the original α-chitin were

mostly maintained after the 33% NaOH treatment, indicating that the partial deacetylation selectively took place on the α-chitin crystallite surfaces. The α-chitin nano-whiskers obtained had average width and length of 6.2±1.1 nm and 250±140 nm, respectively. Especially, long and individual α-chitin nano-fibrils approximately 1 μm in length were detected with the predominant nano-whiskers in the dispersions, which was first observed for crab and shrimp α-chitin fibrils. Because the nano-whisker conversion can be achieved in water at pH 3-4, protonation of the C2-NH_2 groups in the partially deacetylated chitins to provide cationic charges on the crystalline fibril surfaces in high densities, associated with partial mechanical scission of the fibrils during disintegration, is the key driving force for the individualization of α-chitin fibrils. The partially deacetylated α-chitin nano-whiskers dispersed in water at pH 3-4 showed somewhat different rheological properties, and had high light transmittance and viscosity, resulting from individualization of fibrils at high level.

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Chapter 6
Summary

6.1 Preparation of nano-dispersed chitins by TEMPO-mediated oxidation

TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl radical)-mediated oxidation was applied to crab shell α-chitins and tubeworm β-chitins. When the TEMPO-oxidized α-chitins and tubeworm β-chitins were subjected to ultrasonication in water, the slurries were converted to highly viscous and translucent gels, which consisted of mostly individualized chitin nano-whiskers (average width 8 nm and average length 340 nm) and nano-fibers (width 20-50 nm and at least several microns in length), respectively. In these cases, the position-selective formation of dissociated C6 carboxylate groups on the chitin nano-crystallite surfaces by TEMPO-mediated oxidation is the necessary point to convert original chitins to individualized nano-whiskers or nano-fibers. Electrostatic repulsions and/or osmotic effects between anionically-charged chitin microfibrils are likely to be the key driving force for the nano-conversion. Therefore, the mechanism to prepare chitin nano-fibers/whiskers is intrinsically the same as that for preparation of cellulose nano-fibers by TEMPO-mediated oxidation of native celluloses reported by Saito et al.

6.2 Preparation of nano-dispersed chitins by mechanical treatment under acid conditions

6.2.1 Preparation of squid pen β-chitin nano-fibers. Based on the mechanism of the TEMPO-mediated oxidation method to prepare cellulose and chitin nano-fibers/whiskers, cationization of chitin microfibrils were studied. When squid pen β-chitin was disintegrated in water at pH 3-4 for several minutes by ultrasonication, highly viscous and transparent gels were obtained. The TEM (Transmission Electron Microscope) observations revealed that the gels consisted of nano-fibers 3-4 nm in cross sectional width and at least a few microns in length. Cationization or protonation of the C2 amino groups present on the crystalline fibril

surfaces under acid conditions is likely to be one of the most significant and necessary conditions for the nano-fiber conversion. Thus, nano-fibers can be directly obtained from squid pen β-chitin at pH 3-4 without any chemical modification. Due to the high aspect ratios of individualized squid pen β-chitin nano-fibrils (3-4 nm in width and at least a few microns in length with aspect ratios of more than 500), transparent films with high strength or sponge-like aerogels after freeze-drying were obtained.

6.2.2 Individual chitin nano-whiskers prepared from partially deacetylated α-chitins. When partially deacetylated α-chitins were disintegrated in water at pH 3-4 by magnetic stirring for several days, highly viscous and transparent gels consisting of mostly individual rod-like nano-whiskers 6-7 nm and 100-500 nm in width and length respectively, were obtained. This is the first finding to obtain α-chitin nano-whiskers having widths quite similar to the crystal sizes determined by X-ray diffraction method. Thus, complete individualization of α-chitin fibrils can be achieved by the partial deacetylation and the following disintegration in water at pH 3-4. It should be noted that some long and individual nano-fibers with length up to microns were observed in the TEM images, which was also revealed in this study. Partial conversion of the C2 acetylamide groups present on the crystallite surfaces of α-chitin to the corresponding amino groups by partial deacetylation and the following cationization/protonation under acid conditions is necessary for nano-fibrillation of α-chitin, providing strong inter-fibrillar electrostatic repulsions. The partially deacetylated α-chitin nano-whiskers dispersed in water at pH 3-4 showed somewhat different rheological properties, and had high light transmittance and viscosity, resulting from individualization of fibrils at the high level.

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范　一民 (FAN YIMIN)

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Conference

International conference

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