Carbohydrate Polymers 79 (2010) 1046–1051

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Individual chitin nano-whiskers prepared from partially deacetylated α-chitin by fibril surface cationization

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ARTICLE INFO

Article history:
Received 2 September 2009
Received in revised form 29 September 2009
Accepted 15 October 2009
Available online 22 October 2009

Keywords:
Chitin
Nano-whisker
Partial deacetylation
Individualization
Chitin fibril

ABSTRACT

α-Chitin was partially deacetylated to degrees of *N*-acetylation (DNAc) 0.74–0.70 by 33% NaOH treatment at 90 °C for 2–4 h. Solid recovery ratios or yields of the products were 85–90%. Crystallinity index and crystal size of the original α-chitin were maintained, showing that the partial deacetylation mostly occurred on the α-chitin crystallite surfaces. Transparent and highly viscous liquids were obtained by disintegration of the partially deacetylated chitins in water at pH 3–4. Transmission electron microscopy (TEM) revealed that the liquids consisted mostly of individual nano-whiskers with average width and length 6.2 ± 1.1 and 250 ± 140 nm, respectively. Some α-chitin nano-fibrils of more than 500 nm in length were also detected in TEM images. Because the DNAc values 0.74–0.70 correspond to 1.34–1.56 mmol g^{-1} C2-amino group contents, high cationic charges might be formed on the α-chitin fibril surfaces in high density by protonation in water at pH 3–4.

1. Introduction

Native chitins consist of crystalline fibrils, whose lateral dimensions range from 2.5 to 25 nm, depending on their biological origins (Watthanaphanit, Supaphol, Tamura, Tokura, & Rujiravanit, 2008), and chitins have the potential to be converted to individual nano-fibrils by some downsizing processes. However, it is generally difficult to achieve complete individualization of chitin fibrils, because the fibrils are tightly bonded to each other through a large number of hydrogen bonds to physically and biologically support living bodies. When harsh mechanical treatments such as high energy ultrasonication were applied to crab and shrimp shell α-chitins for 30–45 min, bundles of fibrils 30–120 nm in width were obtained (Zhao, Feng, & Gao, 2007). Preparation methods for α-chitin nano-crystals have been already established: a representative protocol consists of hydrolysis of α-chitin with 3 M HCl at high temperatures, which is accompanied by sacrifice of yield and degree of polymerization (Goodrich & Winter, 2007; Gopalan Nair & Dufresne, 2003a; Li, Revol, & Marchessault, 1996b, 1997; Li, Revol, Naranjo, & Marchessault, 1996a; Lu, Weng, & Zhang, 2004; Paillet & Dufresne, 2001). These chitin nano-crystals with spindle-like morphologies 10–12 nm in width and approximately 200 nm in length have been studied as nano-composite materials for reinforcement, for nanoscaffolds in tissue engineering and for other applications (Gopalan Nair & Dufresne, 2003a, 2003b, 2003c; Junkasem, Rujiravanit, & Supaphol, 2006; Morin & Dufresne, 2002; Muzzarelli et al., 2007; Phongying, Aiba, & Chirachanchai, 2007). Since a large amount of crab and shrimp shell chitins are produced as food wastes every year, there is a need for versatile utilization of α-chitins as functional materials.

We have developed new methods to prepare mostly individual and highly crystalline cellulose nano-fibrils and chitin nano-crystals dispersed in water by 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO)-mediated oxidation of native celluloses and chitins, and subsequent mild mechanical agitation in water (Fan, Saito, & Isogai, 2008a, 2009; Saito, Kimura, Nishiyama, & Isogai, 2007; Saito, Nishiyama, Putaux, Vignon, & Isogai, 2006; Saito et al., 2009). Formation of anionically charged C6-carboxylate groups in high density on the cellulose and chitin crystallite surfaces by TEMPO-mediated oxidation is the key driving force for conversion to individual cellulose nano-fibrils and chitin nano-crystals dispersed in water.

Based on the above principle, individual chitin nano-fibrils 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 water at pH 3–4 (Fan, Saito, & Isogai, 2008b). In this case, cationic charges were formed at the glucosamine units on the fibril surfaces, which then brought about interfibrillar electrostatic repulsion in water. This simple disintegration method for preparation of nano-fibrils of squid pen β-chitin is advantageous in terms of safety issues, for application in functional

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doi:10.1016/j.carbpol.2009.10.044

foods, cosmetics and related fields, because the protocol involves no chemical modification. However, this method of individualization of fibrils was not applicable 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 specificity (Fan et al., 2008b).

α-Chitins are more abundantly present in nature in shells of crab, shrimp, etc., and purified α-chitins are commercially more readily available, compared with squid pen β-chitin. Hence, α-chitins rather than β-chitins are preferable resources for individualization to nano-fibrils. Grinding α-chitin in water at pH 3 provided fibril bundles 10–20 nm in width (Fan et al., 2008b; Ifuku et al., 2009). Based on X-ray diffraction patterns of α-chitins, the widths of individual fibrils should be 6–10 nm. Efforts are, therefore, still needed to obtain completely individualized α-chitin nano-fibrils in high yield without any additional chemical modifications.

In this study, partial deacetylation was applied to a commercial α-chitin to selectively increase the C2-primary amino groups on the crystalline fibril surfaces. If the cationic charge density on the crystalline fibril surfaces of α-chitin can be raised, individualization may be achieved by enhanced electrostatic repulsion between cationically-charged fibrils with high charge 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 a decrease in crystallinity (Li et al., 1997). Thus, other conditions are needed 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 (Kurita, Sannan, & Iwakura, 1977).

2. Materials and methods

2.1. Materials

Purified α-chitin powder from crab shell was a commercial product (TCI Laboratory Chemicals Co., Japan) with inhomogeneous morphology. The long and short axis lengths of the α-chitin particles were 76 ± 40 and 56 ± 30 μm, respectively. Sodium hydroxide, sodium borohydride and other chemicals and solvents were laboratory grade (Wako Pure Chemicals Co., Japan), and used as received.

2.2. Partial deacetylation

α-Chitin (1 g) was suspended in 33% (w/w) NaOH (25 ml) containing 0.03 g $NaBH_4$ to prevent alkali-induced depolymerization and weight loss, and the slurry was heated at 90 °C for 1–4 h with shaking every 15–20 min. The partially deacetylated chitin was collected, and thoroughly washed with de-ionized water by repeated centrifugation at 4100*g* for 15 min to neutrality. A portion of the wet NaOH-treated product was freeze-dried for further analyses, and the rest was kept in the wet state at 4 °C.

2.3. Determination of degree of N-acetylation (DNAc)

DNAc values of the original and NaOH-treated chitins were calculated from both cationic charges determined by electric conductivity titration method, and nitrogen/carbon contents by elemental analysis using a Thermo Finnigan Flash EA1112 (Fan et al., 2008a, 2009; Li et al., 1996b; Saito et al., 2006, 2007, 2009). For cationic charge determination, water (60 ml) and a small amount of 0.5 M NaOH to adjust the pH to 9 were added to a dried sample (0.1 g). The mixture was stirred for 30 min to prepare a well dispersed slurry, then 0.1 M HCl was added to adjust the pH to 2.5–3.0, and 0.05 M NaOH solution was added at 0.1 ml min^{-1} up to pH 11 using a pH-stat titration system. The conductivity and pH curves obtained reflected the content of C2-amino groups in the chitins (Li et al., 1996b).

2.4. X-ray diffraction analysis (XRD)

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. XRD patterns were recorded in the range $5° \leqslant 2\theta \leqslant 35°$ 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 NaOH-treated products obtained in this study had XRD patterns of the α-chitin type, the crystallinity index of each sample was calculated from the XRD pattern according to the reported method (Fan et al., 2008; Li et al., 1997). The crystal sizes of the (0 2 0) and (1 1 0) planes were measured from the width at half-height of the original and deconvoluted diffraction peaks at 9.6° and 19.6°, respectively, using Scherrer's equation (Alexander, 1979).

2.5. Mechanical disintegration

The partially deacetylated chitins in the wet state were suspended in water at 0.1% (w/v) solid content. 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. 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, Nihonseiki Co., Japan).

2.6. Analyses of the dispersion

The chitin dispersions at 0.1% solid content, described above, were contained in a poly(methyl methacrylate) disposable cuvettes, and their transmittance measured from 300 to 750 nm using a Shimadzu UV-1700 UV–vis spectrophotometer. The spectrum of a cuvette filled with water was used as reference to correct the transmittance of the dispersion. A 10 ml aliquot of a 0.02% (w/v) sample/water dispersion was mounted on a glow-discharged carbon-coated electron microscopy grid. After approximately 1 min, the excess liquid was absorbed with filter paper, and one drop of 2% uranyl acetate negative stain was added before drying. Excess solution was removed with a filter paper, and the sample allowed to dry by natural evaporation. The sample grid was observed at 100 kV using a JEOL transmission electron microscope (JEM 2000-EXII). All micrographs were collected with a charge coupled device (CCD) camera (Keen View, Olympus Soft Imaging Solutions, Germany) and recorded using iTEM® software supplied by the same company.

3. Results and discussion

3.1. Partial deacetylation of α-chitin

Fig. 1 shows the relationship between the treatment time of α-chitin with 33% NaOH at 90 °C and DNAc of the products. DNAc was reduced from 0.90 to 0.77 by NaOH treatment for 1 h, then gradually decreased after 4 h to 0.70 as determined by conductivity titration. The DNAc value determined by elemental analysis was similar to that determined by conductivity titration at each time, although some discrepancies were observed in DNAc values determined with the two methods. The conductivity titration might have preferentially detected primary amino groups present on

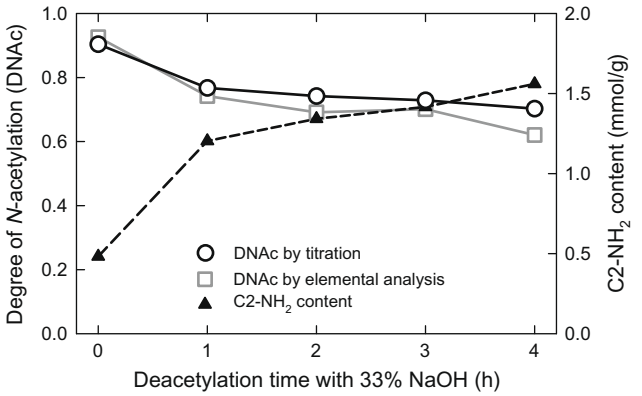

Fig. 1. Relationship between 33% NaOH treatment time of α-chitin at 90 °C and either degree of *N*-acetylation (DNAc) or C2-amino group content in the products. DNAc values were determined by conductivity titration and elemental analysis, and the C2-NH_2 contents were calculated from the DNAc values determined by conductivity titration.

the outer surfaces of α-chitin fibrils (Li et al., 1997). The C2-primary amino group contents, calculated from DNAc values obtained by conductivity titration, are also plotted in Fig. 1. The C2-NH_2 group content increased from 0.48 to 1.56 mmol g^{-1} by NaOH treatment for 4 h. Hence, when the C2-NH_2 groups are protonated under acid conditions, significant amounts of cationic charges can 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 products were lost by handling during the repeated centrifugation treatments, high solid recovery ratios 85–90% were maintained for all of the products obtained.

Crystal structures of the 33% NaOH-treated products were investigated via their XRD patterns (Fig. 2). All partially deacetylated chitins had typical diffraction patterns of α-chitin, and the original α-chitin structure was maintained during NaOH treatment for up to 4 h. The crystal sizes of the (0 2 0) and (1 1 0) planes and crystallinity indices were calculated from the XRD patterns (Fig. 3). The crystal sizes 9.6 and 6.7 nm for (0 2 0) and (1 1 0) planes, respectively, and crystallinity index 0.59 of the original α-chitin

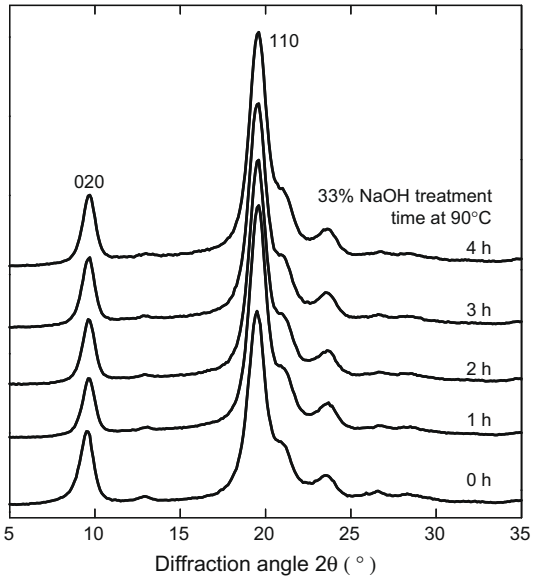

Fig. 2. X-ray diffraction patterns of the original and partially deacetylated chitins prepared by 33% NaOH treatment at 90 °C for 1–4 h.

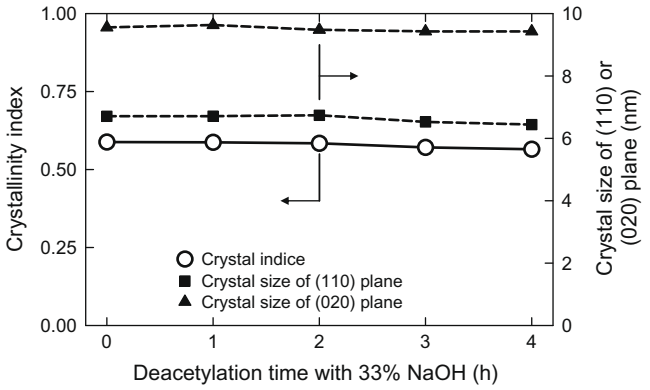

Fig. 3. Crystallinity indices and crystal sizes of the (0 2 0) and (1 1 0) planes of partially deacetylated chitins prepared by 33% NaOH treatment at 90 °C for 1–4 h.

were slightly decreased to 9.4, 6.4 and 0.57 nm, respectively, by 33% NaOH treatment for 4 h. These results demonstrate that partial deacetylation with 33% NaOH at 90 °C for 1–4 h mostly takes place on the surfaces of α-chitin crystallites. Distribution of anhydroglucosamine units on crystallite surfaces of the partially deacetylated α-chitins is a significant research subject of our future work.

When 40% NaOH at 90 °C was used in the deacetylation treatment, partial decrystallization was observed for the products, indicating that deacetylation occurred also inside α-chitin crystallites. On the other hand, it took a longer time to obtain partially deacetylated chitins with the same DNAc values, when 25% NaOH at 90 °C was adopted. Thus, the 33% NaOH treatment was suitable and effective for partial and position-selective deacetylation of α-chitin crystallite surfaces.

3.2. Disintegration of partially deacetylated chitins in water

When the partially deacetylated chitins with DNAc 0.74–0.70 were disintegrated in water at pH 3–4, they were changed to highly viscous and transparent liquids (Fig. 4). Solution state ^{1}H- and ^{13}C-NMR spectra revealed that almost no deacetylated α-chitin molecules were present as a soluble fraction at molecular level in the liquids (data not shown). Thus, the transparent liquids 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 converted to transparent liquids; cationization or protonation of the C2-NH_2 groups at pH 3–4 was required for conversion of the partially deacetylated chitins to transparent liquids by mechanical disintegration in water. The pH titration curves showed that pK_b of the C2-NH_3^+ of partially deacetylated chitins was approximately 6, which supported the above cationization mechanism. The original α-chitin with DNAc of 0.90 was slightly swollen in water at pH 3–4 after extended stirring time or much harsh disintegration treatments using ultrasonic and mechanical homogenizers. However, no transparent liquids could be obtained (Ifuku et al., 2009), 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 solid contents of more than 0.1%. The optical anisotropy indicated that the transparent liquids with high viscosities even at low solid contents consisted of chitin fibrils dispersed at nano level.

In this experiment, we adopted agitation of slurries using magnetic stirrer bars for gentle mechanical treatment for 5 days and subsequent ultrasonication for 1 min to obtain transparent liquids.

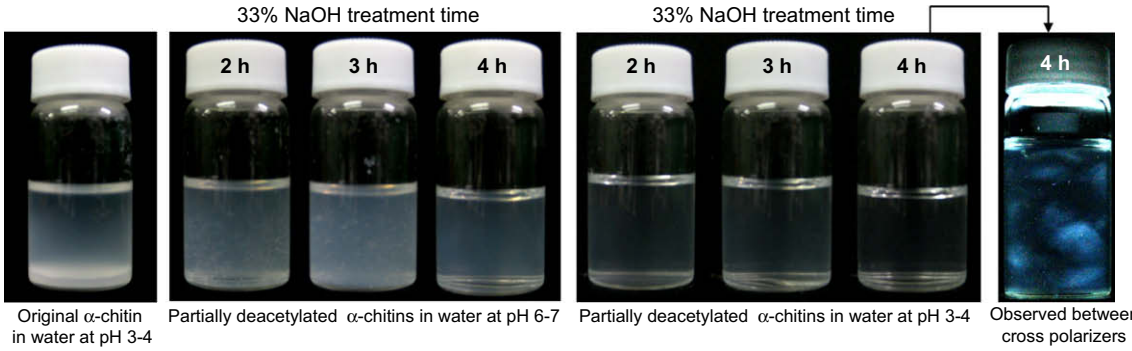

Fig. 4. Dispersion states of the original and partially deacetylated α-chitins in water at various pHs.

When a combination of ultrasonic and double cylinder-type homogenizers was used for disintegration in water at pH 3–4, similar transparent liquids were obtained within 10 min. This harsh mechanical treatment might have brought about some damage to chitin molecules and fibrils.

TEM observations revealed that the transparent liquids consisted of rod-like nano-whiskers, showing that individualization of α-chitin fibrils was achieved by partial deacetylation with 33% NaOH at 90 °C for 3 h and subsequent mechanical disintegration in water at pH 3–4 (Fig. 5). It is noteworthy that some long individual fibrils with widths similar to those of the whiskers but lengths of more than 500 nm were observed in the TEM images; these fibrils have not been previously reported for crab or shrimp shell α-chitin fibrils. Position-selective partial deacetylation in combina-

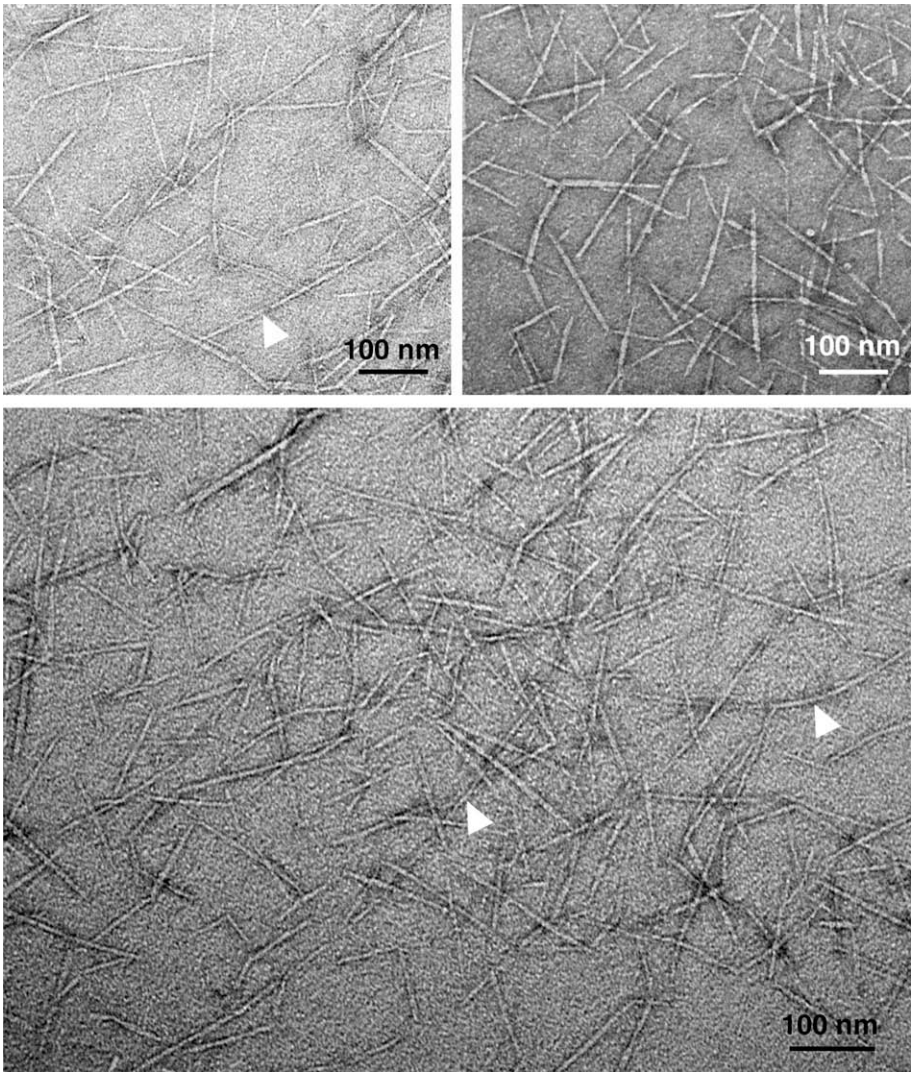

Fig. 5. TEM images of partially deacetylated chitin with DNAc 0.73 prepared from α-chitin by 33% NaOH treatment at 90 °C for 3 h followed by disintegration in water at pH 3–4. Arrows indicate the presence of long fibrils.

tion with gentle agitation of the slurries in water at pH 3–4 might have resulted in effective separation and individualization of α-chitin fibrils with less mechanical damages.

For a sample of approximately 100 individual whiskers in TEM images (Fig. 6), the average length and cross-sectional width were 250 ± 140 and 6.2 ± 1.1 nm, respectively. The nano-whisker widths measured from TEM images roughly corresponded to the crystal size of the (110) plane determined from XRD pattern of the same sample (6.4 nm, Fig. 3), whereas the crystal size of the (0 2 0) plane (9.4 nm in Fig. 3) was larger than the nano-whiskers widths. Even though some discrepancy between the crystal size of the (0 2 0) plane and nano-whisker widths observed in TEM images was recognized, the nano-whiskers were mostly present as individual elements, not bundles, dispersed in water.

Although the widths had a narrow distribution, the lengths were widely distributed without any periodicity. Individualization of the partially deacetylated α-chitin fibrils in water at pH 3–4 as nano-whiskers or nano-fibrils can be achieved due to the following two factors. A sufficient amount of protonated surface C2-NH_2 groups of the partially deacetylated chitin fibrils in water at pH 3–4, brings about electrostatic repulsion between cationically-charged α-chitin fibrils. The other factor is mechanical disintegration in water, which is associated with partial chain scission occurring along the fibrils.

Because the nano-whiskers obtained had various lengths without any periodicity, it is not plausible that mechanical scission of the fibrils took place at mechanically weak regions periodically present along the longitudinal direction of each α-chitin fibril. It is more likely that each fibril sustains strong mechanical stress at various positions along the longitudinal direction of each fibril during disintegration in water, resulting in the wide, not-periodical distribution of the nano-whisker lengths.

The UV–vis transmittance of the 0.1% dispersion of the partially deacetylated chitin is depicted in Fig. 7, showing that a highly transparent dispersion consisting of α-chitin nano-whiskers was obtained. The transmittance at 500 nm was 92%, whereas acid hydrolyzed α-chitin (Goodrich & Winter, 2007) and TEMPO-oxidized α-chitin (Fan et al., 2008a) had transmittance <80%. Thus, the individualization of α-chitin nano-whiskers in the dispersions achieved in this study can be regarded as the highest level of separation. Introduction of cationic charges in high density on the crystalline fibril surfaces might have brought about such transparent dispersions consisting of mostly individualized α-chitin nano-whiskers. The high UV–vis transmittance was unchanged for at least two months after preparation of the dispersion. Thus, the partially deacetylated chitin nano-whiskers can maintain the stable dispersion state without peeling-off of the partially deacetylated chitin molecules from the surface of the crystallites in water at pH 3–4 for at least two months.

Some preparation methods for α-chitin nano-whiskers and β-chitin nano-fibrils and their characteristics have been already reported. Representative procedures are: (1) hydrolysis of α-chitin with 3 M HCl and subsequent homogenizer treatment for 30–45 min in water (Goodrich & Winter, 2007); (2) deacetylation of α-chitin with 50% NaOH followed by acid hydrolysis with 3 M HCl (Li et al., 1997); (3) TEMPO-mediated oxidation of α-chitin under suitable conditions and subsequent disintegration in water (Fan et al., 2008a); (4) simple disintegration of squid pen β-chitin in water at pH 3–4 (Fan et al., 2008b). The advantageous and characteristic features of the newly developed α-chitin nano-whiskers are; (1) commercially available pure α-chitins (originating from crab and shrimp shell) can be used as the starting materials; (2) nano-whiskers are obtained in high yields (85–90%); (3) the rod-like morphology of the nano-whiskers supports the high yields, because α-chitin nano-whiskers prepared by acid hydrolysis or TEMPO-mediated oxidation have spindle-like morphologies with a larger distribution range of widths (Fan et al., 2008a; Goodrich & Winter, 2007; Gopalan Nair & Dufresne, 2003a; Li, Revol, Naranjo, & Marchessault, 1996a; Li et al., 1996b, 1997; Lu et al.,

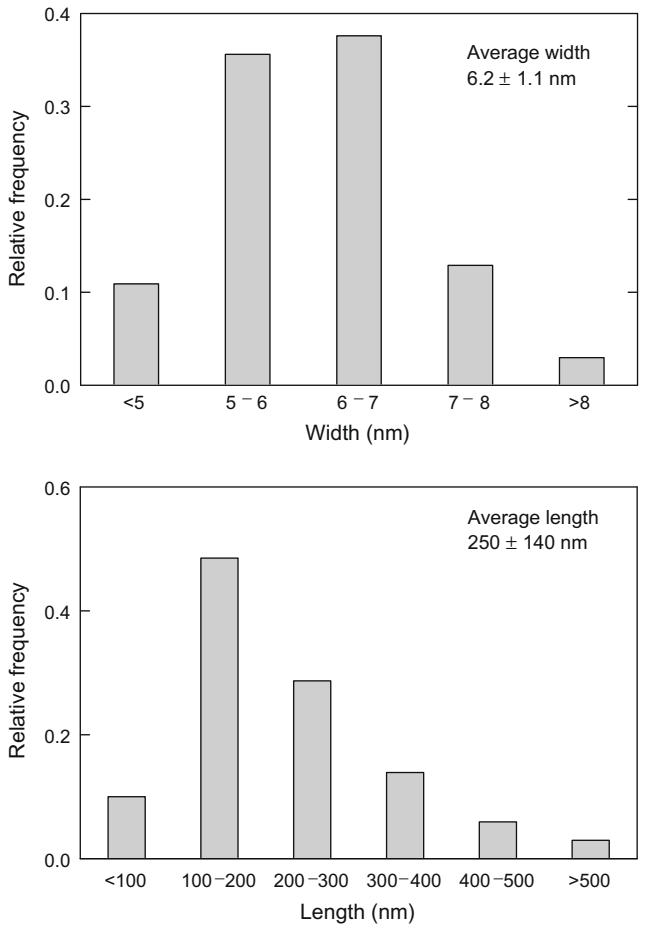

Fig. 6. Distribution of width and length of partially deacetylated α-chitin nano-whiskers prepared by 33% NaOH treatment for 3 h and subsequent disintegration in water at pH 3–4, measured from TEM images.

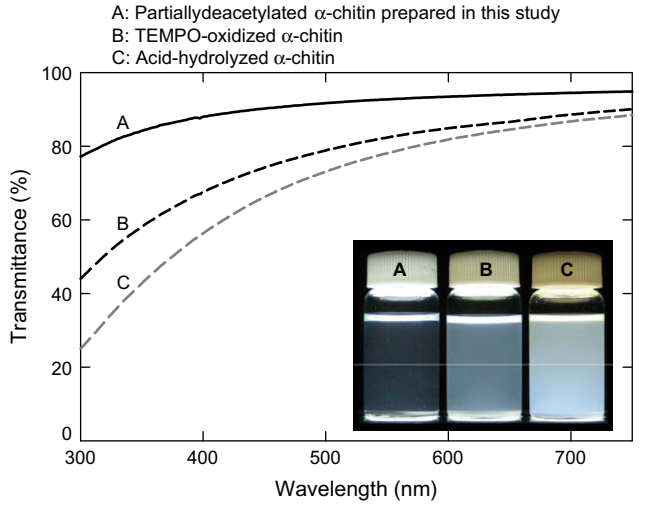

Fig. 7. UV–visible transmittance of 0.1% dispersions at pH 3–4 of partially deacetylated α-chitin with DNAc 0.73. TEMPO-oxidized α-chitin (Fan et al., 2008a) and 3 M HCl-treated α-chitin (Goodrich & Dufresne, 2007) dispersions at the same solid content are also depicted for reference.

2004; Paillet & Dufresne, 2001); (4) the α-chitin nano-whisker dispersions developed in this study had high UV–vis transmittance hence high transparency, indicating that individualization of α-chitin fibrils at a high level was achieved; (5) the α-chitin nano-whiskers obtained in this study have a lower hurdle in terms of safety issues, compared with chemically modified materials such as TEMPO-oxidized α-chitins, hence potential applications can be expanded to not only functional materials but also functional foods, life science and medical fields.

4. Conclusions

Mostly individualized α-chitin nano-whiskers were obtained in yields of 85–90% by partial deacetylation with 33% NaOH at 90 °C for 2–4 h and subsequent disintegration in water at pH 3–4. The DNAc values of the products were 0.74–0.70, which correspond to C2-NH_2 contents of 1.34–1.56 mmol g^{-1}. X-ray diffraction analysis showed that the crystallinity and crystal size of the original α-chitin were mostly maintained after the 33% NaOH treatment, indicating that partial deacetylation takes place selectively on the α-chitin crystallite surfaces. The α-chitin nano-whiskers obtained had average width and length, 6.2 ± 1.1 and 250 ± 140 nm, respectively. In addition, individual α-chitin nano-fibrils of more than 500 nm in length were detected, for the first time for crab and shrimp shell α-chitin fibrils, in dispersions with the predominant nano-whiskers. Because conversion to nano-whiskers was achieved in water at pH 3–4, protonation of the C2-NH_2 groups in the partially deacetylated chitins to provide cationic charges in high density on the crystalline fibril surfaces, associated with partial mechanical scission of the fibrils during disintegration, is the key driving force for the individualization of α-chitin fibrils.

Acknowledgements

This research was supported by Grant-in-Aids for Scientific Research (Grant numbers 18380102 and 18-10902) from the Japan Society for the Promotion of Science (JSPS). Y.M.F. is a recipient of the Monbu-Kagakusho Fellowship for foreign students.

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