Chitin nanocrystals prepared by oxidation of α-chitin using the O₂/laccase/TEMPO system

Jie Jiang a, Wenbo Ye b, Juan Yu a, Yimin Fan a,⁎, Yuko Ono b, Tsuguyuki Saito b, Akira Isogai a,⁎

a Jiangsu Co-Innovation Center of Efficient Processing and Utilization of Forest Resources, Jiangsu Key Lab of Biomass-Based Green Fuel & Chemicals, College of Chemical Engineering, Nanjing Forestry University, Nanjing 210037, China
b Department of Biomaterials Science, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan

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Laccase mediator oxidation was applied to chitin at pH 6.8 and 30 °C to prepare chitin nanocrystals with a catalytic amount of 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO). When 40 mM TEMPO and a total of 500 U laccase were added to 1 g chitin, the yield of water-insoluble oxidized chitin was more than 95%, and the carboxylate content was 0.43 mmol/g. Adsorption of laccase molecules on chitin particles occurred in a buffer at pH 6.8, which may have been caused by electrostatic interactions between positively charged C2-ammonium groups of chitin and anionically charged groups of laccase. Rod-like chitin nanocrystals (ChNCs) were obtained with average lengths and widths of 480 ± 200 nm and 24 ± 17 nm, respectively, by sonication of the oxidized chitin/water suspensions. The O₂/laccase/TEMPO oxidation caused no decrease in the degree of N-acetylation or the crystallinity of the original chitin based on FTIR and X-ray diffraction data.

1. Introduction

Chitin, the second most abundant natural biopolymer, consists of N-acetylgalactosamine and and nhydroxyacetic acid units in various ratios linked by β-(1→4)-glycoside bonds and presents as a supporting and protecting material in the exoskeleton of arthropods and the cell walls of fungi and yeast (Rinaudo, 2006). Similar to cellulose, chitin molecules form highly crystalline fibrils with widths of 3–50 nm, which are embedded in protein and CaCO₃ matrix (Ikekubor, 2014; Raabe, Sachs, & Romano, 2005; Revol & Marchessault, 1993). Since shells of arthropods have hierarchical structures, chitin has the potential to be converted to individual nanocrystals by downsizing. Several routes have been developed to prepare chitin nanocrystals (ChNCs), such as mechanical disintegration, acid hydrolysis, and TEMPO-mediated oxidation (Fan, Saito, & Isogai, 2007; Herrera, Salaberria, Mathew, & Oksman, 2016; Salaberria, Fernandez, Diaz, & Labidi, 2015). The mechanical treatments of chitin using, for instance, a high-pressure homogenizer, convert chitin particles into nanosized fibrils with widths < 100 nm, but this process needs high energy (Salaberria et al., 2015). The acid hydrolysis of chitin with 3 M HCl at high temperatures causes remarkable depolymerization. The obtained nanocrystals are rod-like with widths and lengths of 10–20 nm and < 800 nm, respectively, depending on the chitin origins (Goodrich & Winter, 2007; Paillet & Dufresne, 2001; Rubenthaler, Ward, Chee, & Tang, 2015).

In TEMPO-mediated oxidation, chitin is suspended in water at pH 10 containing TEMPO, sodium bromide and a certain amount of sodium hypochlorite. Significant amounts of sodium C₆-carboxylate groups are formed on the surface of chitin fibrils when chitin is oxidized under suitable conditions. These TEMPO-oxidized chitins are converted to individually dispersed chitin nanocrystals with an average width and length of 8 nm and 340 nm, respectively, by sonication in water, in which electrostatic repulsion efficiently works between anionically charged chitin nanocrystals (Fan et al., 2007; Saito, Kimura, Nishiyama, & Isogai, 2007). Because chitin has abundant C₂-acetamide groups, partial C₂-deacetylation of crab shell chitin leads to the formation of additional amounts of C₂-amine groups on the surface of the chitin. These C₂-amine groups of chitin turn to C₂-ammonium groups with cationic charges in water at pH 2–4 through protonation. Thus, surface- and cell-oxidized ChNCs with lengths and widths of ~ 250 nm and ~ 6 nm, respectively, can be prepared from the partially decacylated chitins by mechanical disintegration in water in an acidic condition (Fan, Saito, & Isogai, 2008, 2010). The positively or negatively charged ChNCs dispersions transformed to homogeneous and transparent hydrogels by gas coagulation of ammonia or hydrochloric acid, respectively (Liu et al., 2016).

Recently, a new system has been developed to oxidize cellulose using laccase and TEMPO (Aracri, Vidal, & Ragauskas, 2011; Aracri, Valls, & Vidal, 2012; Patel, Ludwig, Haltrich, Rosenau, & Potthast, 2015).
The enzyme laccase is a glycosylated oxidase that contains four copper atoms in the active site (Thurston, 1994). Laccase can catalyze the one-electron oxidation of substrates such as phenol, polyphenol, anilines and aliphatic amines to the corresponding radicals in the presence of molecular oxygen (Baldrian, 2006; Mayer & Staples, 2002; Solomon, Sundaram, & Machonkin, 1996). The O₂/laccase system cannot oxidize nonphenolic groups with high redox potentials (> 0.8 V). Moreover, large molecules, such as chitin, cannot directly enter the active site of laccase. Thus, a suitable mediator is required for oxidation of chitin when using the O₂/laccase system, in which the mediator acts as an electron shuttle. The mediator oxidized by O₂/ laccase diffuses away from the active site of laccase and, in turn, oxidizes the C₆-OH groups of chitin in the O₂/laccase/mediator system (Astolfi et al., 2005; Galli & Gentili, 2004).

TEMPO has been reported to act as a mediator in the O₂/laccase system, and the O₂/laccase/TEMPO system has been applied to hydroxy groups of low-molecular-mass compounds and sugars (Arends, Li, Ausan, & Sheldon, 2006; Fabbrini, Galli, Gentili, & Macchitella, 2001; Kędziora, Díaz-Rodríguez, Lavandera, Gotor-Fernández, & Gotor, 2014). Recently, the O₂/laccase/TEMPO system has been used to oxidize cellulose fibers to introduce C₆-aldehyde groups, which play a significant role in improving wet and dry strengths of prepared paper sheets by the formation of inter-fiber hemiacetal linkages (Aracri et al., 2011, 2012). In a previous paper, we prepared oxidized cellulose nanofibrils using the O₂/laccase/TEMPO system, followed by mechanical disintegration in water, in which ~1 mmol/g C₆-carboxylate groups were formed in the oxidized cellulose (Jiang et al., 2017).

In this study, the O₂/laccase/TEMPO system was applied to chitin to introduce C₆-carboxylate groups to prepare ChNCs by associating with the successive mechanical disintegration without using any chlorine-containing oxidant. The chemical and crystal structures of the original and oxidized chitin prepared under various conditions were investigated in terms of degrees of N-acetylation, crystallinity, and crystal size.

2. Experimental methods

2.1. Materials

Chitin particles, isolated and purified from crab shells, was used as the starting material. TEMPO, 2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and laccase from Aspergillus sp. were purchased from Sigma Aldrich (USA). Other chemicals were laboratory grade (Wako Pure Chemical, Osaka, Japan) and used without further purification.

2.2. Enzyme activity

Laccase activity in solutions was monitored by absorbance at 420 nm using an UV–vis spectrophotometer (JASCO V-670). A solution containing laccase (100 μL) was added to a cuvette containing 0.5 mM ABTS solution (3 mM acetate buffer at pH 4.5). One activity unit (U) was defined as the amount of laccase transforming 1 μmol ABTS to its cation radical per minute.

2.3. O₂/laccase/TEMPO oxidation of chitin

1 g chitin was suspended in a flask containing 100 mL TEMPO solutions of 5–40 mM in 0.1 M acetate buffer at pH 6.8 and 30 °C. Oxidation was started by adding laccase to the chitin/TEMPO suspension and magnetically stirred at 500 rpm. During oxidation, laccase was separately added at 0 h, 24 h, 48 h, and 72 h with the amount of 200 U, 100 U, 100 U and 100 U, respectively. The flask was covered with plastic film with several little holes for providing oxygen from the air. After oxidation, the flask was put in ice water for 30 min, and then the mixture was washed with distilled water by repeated centrifugation at 10,337 × g for 8 times to obtain oxidized chitin as a water-insoluble fraction.

2.4. Determination of carboxylate and aldehyde contents in oxidized chitin

The carboxylate content of oxidized chitin was determined by the electrical conductivity titration method (Saito & Isogai, 2004). Dried sample (0.1 g) was suspended in water (60 mL) and stirred for 30 min to prepare a sufficiently swollen chitin suspension. A total of 0.1 M HCl was added to the suspension to adjust the pH to 2.5–3.0. Then, 0.05 M NaOH was added at the rate of 0.1 mL/min until the pH was 11 using a titration apparatus. The aldehyde content was measured by the same electrical titration after post oxidation of the oxidized chitin with NaClO₂, which selectively oxidizes aldehyde groups to carboxyl groups.

2.5. Mechanical disintegration

A 0.1% (w/v) suspension of oxidized chitin was sonicated with an ultrasonic homogenizer (NISSEI US-300E, Tokyo, Japan) with a tip of 0.74 cm at 500 W and 20 kHz for 10 min with start/stop intervals to avoid temperature increases. The dispersion was centrifuged at 12600 × g for 6 min to remove unfractilled fractions, and ChNCs/water dispersion was obtained as a supernatant.

2.6. Analyses

The oxidized chitins were freeze-dried and pressed to disk pellets (0.1 g each) to record their X-ray diffraction (XRD) patterns from 5 to 30° of diffraction angle 2θ with the reflection method using an XRD apparatus (RINT 200, Rigaku, Tokyo, Japan) at 40 kV and 30 mA. The crystallinity index was calculated from the peak intensity I_{101} at 19.6° and the baseline intensity I_{baseline} at 16.0° (Zhang, Xue, Xue, Gao, & Zhang, 2005). The crystal size of (020) and (110) planes were determined according to the half maximum full width of peaks at 9.6° and 19.6°, respectively (Alexander, 1969; Minke & Blackwell, 1978). The oxidized chitins with protonated carboxyl groups were prepared by soaking them in 0.1 M HCl, repeatedly washing them with water, and then freeze-drying them for FTIR measurement using an instrument (JASCO FT/IR-6100, Tokyo, Japan) in the transmission mode from 400 to 4000 cm⁻¹. The light transmittance spectra of 0.1% (w/v) ChNCs dispersions were recorded in the range of 400–800 nm using an UV–vis spectrophotometer (JASCO V-670, Tokyo, Japan). The ChNCs dispersion was diluted to ~0.01% (w/v) with distilled water, and dropped on a mica substrate and dried at 40 °C. The ChNCs samples were scanned by atomic force microscopy (AFM) (Bruker Multimode, Billerica, USA) using the tapping mode with a standard silicon cantilever in air.

3. Results and discussion

3.1. Adsorption of laccase on chitin particles in buffer at pH 6.8

In the O₂/laccase/TEMPO oxidation of chitin, laccase firstly oxidized TEMPO to the N-oxoammonium compound (TEMPO⁺), which can in turn oxidize primary hydroxyl groups to aldehyde and carboxyl groups and transferred to N-hydroxyl-TEMPO (reduced TEMPO). The activity of laccase affects the efficiency of converting C₆-OH groups of chitin to C₆-carboxylate groups, because laccase is a sensitive biomolecule containing various functional groups (Jiang et al., 2017). The influence of TEMPO and chitin on laccase activity was first examined using ABTS as a model substrate. Fig. 1A shows the change in laccase activity in 0.1 M acetate buffer at pH 6.8 containing chitin particles. The laccase activity sharply decreased from 100% to ~40% of the original activity (2 U/mL) during the first 40 min, when chitin particles were present in the mixture. Approximately 30% of the original laccase activity remained for 90 min after the addition of chitin. In contrast, the original activity of laccase decreased by only 2–4% when stored in a...
buffer at pH 6.8 for 24 h.

Thus, the decrease of laccase activity in the chitin suspension (Fig. 1A) was probably caused by adsorption of laccase molecules on the chitin particles through intermolecular force and electrostatic interactions between cationically charged C2-ammonium groups in chitin and anionically charged groups in laccase in the buffer. This is because no such a decrease in laccase activity was observed when cellulose was used in the same system (Jiang et al., 2017). Commercially available crab shell α-chitins have ∼10% C2-amine groups (the rest ∼90% are C2-acetamide groups) (Fan et al., 2007; Fan, Saito, & Isogai, 2010). Because the 2 U/mL laccase was present in the 0.01 g/mL chitin dispersion in the experiment of Fig. 1A, the adsorption of laccase on chitin under equilibrium conditions was estimated to be ∼140 U/g of chitin, which cannot be ignored. The result in Fig. 1A might indicate that only free laccase molecules without adsorption on chitin particles can participate in the oxidation of C6-OH groups of chitin in the O2/laccase/TEMPO system. Expectedly, the laccase activity decreased with increases in the amount of chitin in the system (Fig. 1B).

### 3.2. Change of laccase activity in the O2/laccase/TEMPO system

The effect of the TEMPO concentration in the mixture containing 2 U/mL laccase and 0.01 g/mL chitin is shown in Fig. 2. The laccase activity sharply decreased within 1 h after the TEMPO addition, and then 20–30% laccase activities were maintained up to nearly 10 h, irrespective of the added amount of TEMPO. These results are explained in terms of two factors: adsorption of laccase molecules on chitin particles, as shown in Fig. 1A and B, and degradation of laccase molecules by TEMPO (Jiang et al., 2017). Approximately ∼20% laccase activity remained after 24 h, when 5 mM TEMPO was used, whereas the laccase activity became almost zero after 24 h with 10–40 mM TEMPO.

### 3.3. Oxidation efficiency and properties of oxidized chitin

Based on the results in Figs. 1 and 2, laccase is not stable in the presence of chitin in the O2/laccase/TEMPO system. Next, a total of 500 U laccase was added separately to the chitin suspension as follows: 200 U at 0 h, and 100 U at 24 h, 48 h and 72 h during the oxidation of chitin. The aldehyde and carboxylate contents of the oxidized chitins prepared with 5–40 mM TEMPO obtained as water-insoluble fractions after reaction for 96 h are shown in Fig. 3. The carboxylate content of the oxidized chitins increased from 0.21 to 0.43 mmol/g when the added amount of TEMPO was increased from 5 to 40 mM. The aldehyde groups were regarded as the intermediated product during oxidation and soon post-oxidized to carboxylate groups, and the aldehyde contents of the oxidized chitins were in the range of 0.06–0.1 mmol/g. The yields of oxidized chitins obtained as water-insoluble fractions were greater than 95%, indicating that the destruction of chitin molecules during the O2/laccase/TEMPO system was not significant. When the oxidized chitin prepared with 20 mM TEMPO was analyzed by size-exclusion chromatography attached to multi-angle laser-light scattering (SEC/MALLS) using 1% lithium chloride/N,N-dimethylacetamide (LiCl/DMAc) as an eluent (Ono, Ishida, Soeta, Saito, & Isogai, 2016), the weight-average molecular mass was 214800, while that of the original chitin was 136200 (Fig. S1 in the Supplemental data). When the TEMPO/NaBr/NaClO system is applied to α-chitin, the yields of oxidized chitins obtained as water-insoluble fractions clearly decreased with the amount of NaClO added (Fan et al., 2007, 2010). Thus, the O2/laccase/TEMPO system in the buffer at pH 6.8 was advantageous in terms of the yield of oxidized chitins, although the carboxylate contents...
of the oxidized chitins prepared in this study were lower than those prepared using the TEMPO/NaBr/NaClO system. All the oxidized chitins were soaked in 0.1 M HCl, followed by thorough washing with water to convert the sodium C6-carboxylate groups to protonated carboxyl groups. These protonated samples were analyzed by FTIR (Fig. 4). The adsorption of protonated carboxyl groups was observed at 1740 cm\(^{-1}\) for the oxidized chitins prepared with 10–40 mM TEMPO. The C–O stretching vibration of chitin skeletons has an absorption peak at 1030 cm\(^{-1}\) and was used as an internal standard for quantitative analysis of carboxyl groups in the oxidized chitins (Shigemasa, Matsura, Sashiwa, & Saimoto, 1996). The absorption ratio of A\(_{1740}/A_{1030}\) corresponded well with the measured carboxylate content of the oxidized chitins, as shown in Fig. 3. The amide II band had an absorption at 1560 cm\(^{-1}\), and the ratio of A\(_{1560}/A_{1030}\) of the original chitin was ∼0.67, which corresponded to the degree of N-acetylation of 0.92 using a calibration line proposed by Shigemasa et al. (1996). The calculated degrees of N-acetylation of the oxidized chitins prepared with 5–40 mM TEMPO were in the range of 0.91–0.92. Thus, almost no deacetylation of C2-acetamide groups in the oxidized chitin occurred during the O\(_2\)/laccase/TEMPO oxidation. The XRD patterns of the original and oxidized chitins are shown in Fig. 5. The characteristic diffraction peaks of α-chitin were detected for all the oxidized chitins at 9.6°, 19.6°, 21.1° and 23.7° of diffraction angle 2θ. The crystallinity and crystal size of the oxidized chitins were almost unchanged by the oxidation. The slight increases in crystallinity of the oxidized chitins were probably caused by partial removal of disordered regions present in the original chitin during oxidation as water-soluble fractions. These results showed that the oxidation of C6-OH groups to C6-carboxylate groups selectively occurred on the crystalline chitin microfibril surfaces.

### 3.4. Characterization of ChNCs dispersions

The oxidized chitins prepared in the above sections were subjected to ultrasonic treatment to prepare oxidized chitin nanocrystals (ChNCs). The carboxylate content of oxidized chitins and celluloses significantly affected their nano-dispersibility by the sonication in water. Nanofibers/nanowhiskers can be prepared by electrostatic repulsion originating from the dissociated C6-carboxylate groups present on the surfaces of oxidized chitin and cellulose microfibrils, which efficiently work in water.

The original chitin particles did not nano-disperse at all in water and were mostly present as sediments in the bottom of the bottle soon after stopping the long-time sonication. When the sonication was applied to the oxidized chitin/water suspensions, they turned to stable dispersions. However, the oxidized chitins prepared with 5 mM and 10 mM TEMPO had high turbidities, which were caused by significant amounts of unbrilliated particles present in the dispersions. The oxidized chitin prepared with 40 mM TEMPO gave a dispersion with higher transparency (Fig. S2).

After centrifugation of the dispersions to remove unbrilliated particles, more transparent ChNCs dispersions were obtained from the oxidized chitins prepared with 5–40 mM TEMPO (Fig. 6) in yields of 43–65% (Table S1). The light transparency of the ChNCs/water dispersions and the yield of ChNCs increased with increases in the carboxylate content of the oxidized chitins. The ζ-potentials of the ChNCs in water are listed in Table S2. The oxidized chitins prepared with greater amounts of TEMPO gave higher carboxylate contents (Fig. 3), resulting in higher negative ζ-potentials of ChNCs. These results showed that ChNCs are dispersed in water by electrostatic repulsion between anionically charged C6-oxidized chitins. The carboxylate contents of the oxidized chitins prepared with 5–40 mM TEMPO ranged from 0.2 to

![Fig. 4. FTIR spectra of oxidized chitin prepared with 5–40 mM TEMPO.](image)

![Fig. 5. XRD patterns of oxidized chitin prepared with 5–40 mM TEMPO.](image)

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<th>Table 1</th>
<th>Crystallinity and crystal size of the original and oxidized chitins prepared with 5–40 mM TEMPO.</th>
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<td>Crystallinity (%)</td>
<td>90.3 92.1 92.1 92.4 93.2</td>
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<tr>
<td>Crystal size (nm)</td>
<td>5.23 5.28 5.27 5.35 5.31</td>
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![Fig. 6. Light transmittance spectra of 0.1% dispersions of ChNCs prepared with 5–40 mM TEMPO.](image)
0.4 mmol/g (Fig. 3), whereas the original chitin had C2-amine groups of ~0.56 mmol/g based on the degree of N-acetylation of 0.9 (in the Supplemental data). The results in Table S2 indicate that almost all C6-carboxylate groups are present as dissociated C6-carboxylate structures, whereas most C2-amine groups were present as non-protonated C2-NH2 structures (without forming the cationized C2-N+H3) in the neutral aqueous dispersions.

The morphology of ChNCs prepared with 20 mM TEMPO was observed by AFM, and a typical AFM image is shown in Fig. 7A. More than 200 registered ChNCs had average lengths and widths of 480 ± 200 nm and 24 ± 17 nm, respectively (Fig. 7B). Approximately 70% of ChNCs had widths of 10–30 nm. Thus, chitin nanorods or nanocrystals were prepared from α-chitin by the O2/laccase/TEMPO oxidation and successive sonication in water.

4. Conclusion

Laccase molecules adsorbed on chitin through intermolecular force and electrostatic interactions due to the amino groups, and TEMPO had a significant influence on the oxidation efficiency. In 40 mM TEMPO solution, when 500 U laccase was added to 1 g chitin, the carboxylate content of the oxidized chitin reached 0.43 mmol/g, and the solid recovery of oxidized chitin was over 95%. During oxidation, no obvious deacetylation and change of crystal size were detected. On the whole, the ChNC dispersions showed high transmittance, and the ChNCs were rod-like with average lengths of 480 ± 200 nm and widths of 24 ± 17 nm.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.carbpol.2018.01.096.

References


