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Effect of sub- and supercritical water pretreatment on enzymatic degradation of chitin

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ABSTRACT

We examined the effect of sub- and supercritical water pretreatment (300–400 °C, 0.5–15 min) on enzymatic degradation of chitin to *N*,*N*′-diacetylchitobiose $(GlcNAc)_2$. The yield of $(GlcNAc)_2$ by enzymatic degradation of supercritical water pretreated chitin at 400 °C for 1.0 min was up to 37%, compared to 5% without the pretreatment. X-ray diffraction (XRD) analysis revealed that the *d*-spacing and the crystallite size increased by sub- and supercritical water pretreatment, which is indicative of swelling of the chitin. The swelling of the chitin crystal structure improved enzymatic degradation by allowing the enzymes easy access to the chitin.

1. Introduction

N,*N*′-Diacetylchitobiose $(GlcNAc)_2$, is a dimer of *N*-acetylglucosamine (GlcNAc). The GlcNAc from crustacean chitin (α-chitin) is a versatile functional compound used in skin moisturizers, analgesics for joint pain, and as antitumoral and antimicrobial agents (Liang, Chen, Yen, & Wang, 2007; Liu et al., 2011; Muzzarelli et al., 2012; Muzzarelli, 2011; Suzuki et al., 1986; Wang, Lin, Yen, Liao, & Chen, 2006; Wang et al., 2008). $(GlcNAc)_2$ has milder and additional physiological activities compared to GlcNAc and it is an attractive building block for the production of oligomers (Nanjo, Sakai, Ishikawa, Isobe, & Usui, 1989; Usui, Matsui, & Isobe, 1990). Although the market for $(GlcNAc)_2$ and GlcNAc is rapidly expanding, their production from crab shells involves numerous steps and requires strong acid because of the crystallinity and the insolubility of α-chitin. In light of the difficulties associated with the traditional production process for production of $(GlcNAc)_2$ and GlcNAc, environmentally compatible and reproducible alternatives for enzymatic degradation are desired. An enzymatic process that depolymerizes chitin completely would be environmentally acceptable since it would avoid the use of deleterious substances and the generation of large amounts of wastewater. However, α-chitin is insoluble in water under ambient conditions and its crystallinity is possibly too high to permit enzymatic depolymerization.

The aim of this study is the production of $(GlcNAc)_2$ from chitin by a combination of sub- and supercritical water pretreatment (Tc = 374.3 °C, Pc = 22.1 MPa) and enzymatic degradation. There are some reports that sub- and supercritical water treatment on its own is not sufficient to obtain $(GlcNAc)_2$ and GlcNAc from chitin because, under these conditions, they decompose at the same time as the chitin is hydrolyzed (Sakanishi, Ikeyama, Sakaki, Shibata, & Miki, 1999; Quitain, Sato, Daimon, & Fujie, 2001; Yoshida, Ehara, & Saka, 2004; Sato, 2004). In this study, we used pretreatment of chitin in sub- and supercritical water for enzymatic degradation and investigated conditions that promote enzymatic degradation without including decomposition of $(GlcNAc)_2$ and GlcNAc.

2. Materials and methods

2.1. Materials and enzymes

Crab chitin (Yaizu Suisankagaku Industry) was used as the starting material. The size of the flaked chitin was about 3 mm × 3 mm × 0.5 mm. For the enzymatic degradation, a crude enzyme extract from *Streptomyces griseus* (Eikon CHL from Rakuto Kasei Industrial) was used as chitinase. For each substrate, we used 90 [U] of enzyme. One unit of the enzyme activity was defined as the amount of enzyme required to release 1 μmol of *N*-acetylglucosamine from ethylene glycol chitin solution in 1 min at 40 °C, pH 6.0. The enzyme produces $(GlcNAc)_2$ as a major product from chitin, which was then hydrolyzed to GlcNAc by chitobiase.

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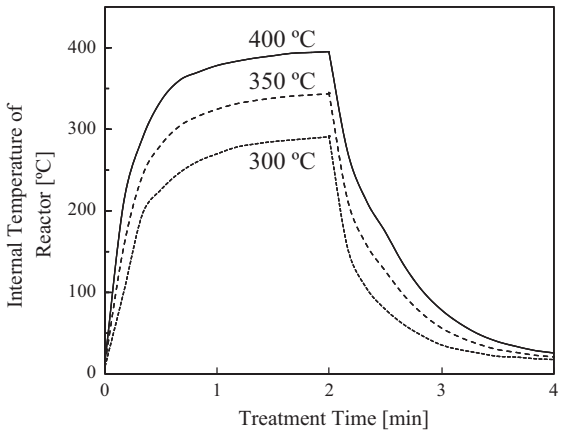

Fig. 1. Internal temperature profile of the reactor (quenched in a water bath at 2 min).

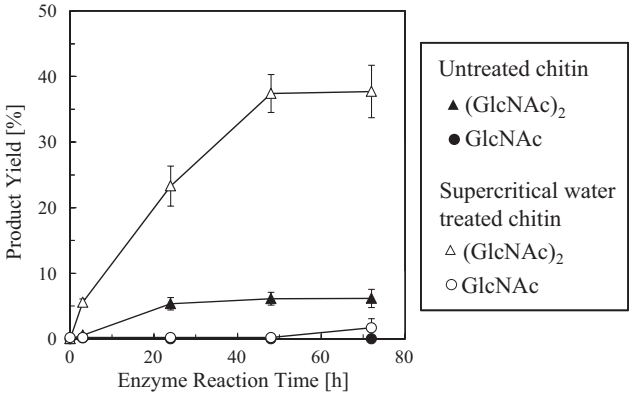

Fig. 2. Reaction time profile for enzymatic degradation of untreated and supercritical water treated chitin at 400 °C for 1 min ($n = 3$, bars indicate SDs).

2.2. Sub- and supercritical water pretreatment

Pretreatment of chitin in sub- and supercritical water was conducted in a stainless steel 316 tube reactor of 6 cm^3 volume. Chitin flakes (0.2 g) and water (3 g) were loaded in the reactor, and the reactor was submerged into a molten-salt bath (KNO_3–$NaNO_3$) kept at 300, 350, and 400 °C reaction temperatures. Fig. 1 shows the internal temperature profile of the reactor at each temperature setting. The reaction start point was set at the moment when the reactor was submerged into the molten-salt bath; therefore, the reaction times include the initial heating period. After a given reaction time, the reactor was removed from the molten-salt bath and rapidly quenched in a water bath to cool down to room temperature. After cooling, the products were collected from the reactor and divided into the water-soluble and solid fractions (chitin) using a membrane filter (pore size: 0.20 μm, Millipore). After sub- and supercritical water pretreatment, the chitin was dried at 90 °C for 24 h.

2.3. Enzymatic degradation of chitin

Enzymatic degradation of chitin was conducted as follows. 20 mg of chitin (1% of final concentration) was mixed with 1.8 mL of 10 mmol/L phosphate buffer (pH 6.0) and 0.2 mL of 10 mg/mL enzyme diluted with 10 mmol/L phosphate buffer (0.1% of final concentration, approximately 100 U). The reaction mixture was shaken at 1400 rpm at 40 °C, and 0.4 mL of the mixture was harvested at the appropriate time. The harvested reaction solution was filtered (pore size 0.45 μm, ADVANTEC) after boiling for 10 min, and centrifuged.

2.4. High-performance liquid chromatography (HPLC)

The HPLC system consisted of a Model 300S ELSD detector (SofTA), and L-2000 system (Hitachi). Products were separated on a Shodex SUGAR KS-802 column (Ø 0.8 cm × 30 cm) using ultrapure water (Millipore) as the mobile phase, at a flow rate of 0.6 mL/min at 60 °C. Yield was calculated from the area of the peak of the enzymatic reaction mixture and two standards of $(GlcNAc)_2$ and GlcNAc. The product yield is defined as given below

$$\text{Product yield (\%)} = \frac{\text{weight of } (GlcNAc)_2 \text{or GlcNAc}}{\text{weight of chitin(20 mg)}} \times 100. \quad (1)$$

2.5. Characterization of the chitin by X-ray diffraction (XRD) and infrared red (IR)

Equatorial diffraction profiles were obtained with Cu-Kα from a powder X-ray generator (Japan Electronic Organization Co. Ltd., JDX-3530) operating at 30 kV and 30 mA. The crystallinity index was calculated from normalized diffractograms according to the equation below. The intensities of the peaks at [1 1 0] lattice (I_{110}, at $2\theta = 20$ corresponding to the maximum intensity of chitin) and I_{am} at $2\theta = 16$ (amorphous diffraction) were used to calculate the crystallinity index (Lavall, Assis, & Campana-Filho, 2007).

$$\text{Crystallinity index (\%)} = \frac{(I_{110} - I_{am})}{I_{110}} \times 100 \quad (2)$$

The d-spacing of the peaks at [1 1 0] lattice was calculated using Bragg's equation

$$2d \sin\theta = \lambda \quad (3)$$

where d is the spacing between the planes in the lattice; θ is the Bragg angle; and λ is the X-ray wavelength.

The crystallite size of chitin at [1 1 0] lattice was calculated using the Scherrer equation

$$L = \frac{0.9\lambda}{(H \cos\theta)} \quad (4)$$

where L is the crystallite size perpendicular to the plane; and H is the full-width at half-maximum in radians.

The FT-IR spectroscopy of the chitin was measured with a Nicolet iS10 spectrometer (Thermo Fisher Scientific Inc.).

3. Results

3.1. Effect of the sub- and supercritical water treatment on enzymatic degradation of chitin

Fig. 2 shows the yield of $(GlcNAc)_2$ and GlcNAc by enzymatic degradation of untreated and supercritical water treated chitin at 400 °C for 1 min. For untreated chitin, the yield of $(GlcNAc)_2$ at 72 h was only 5%. The yield of $(GlcNAc)_2$ after treatment with supercritical water was 37% at 72 h. Fig. 2 indicates that the enzymatic degradation was completed by 48 h. The yields of GlcNAc by enzymatic degradation at 72 h of untreated and supercritical water treated chitin were only 0 and 1.7%, respectively, therefore we focused our analysis on $(GlcNAc)_2$. We also analyzed the aqueous solution recovered after supercritical water treatment without subsequent enzymatic treatment; however $(GlcNAc)_2$, and GlcNAc were not obtained under these conditions.

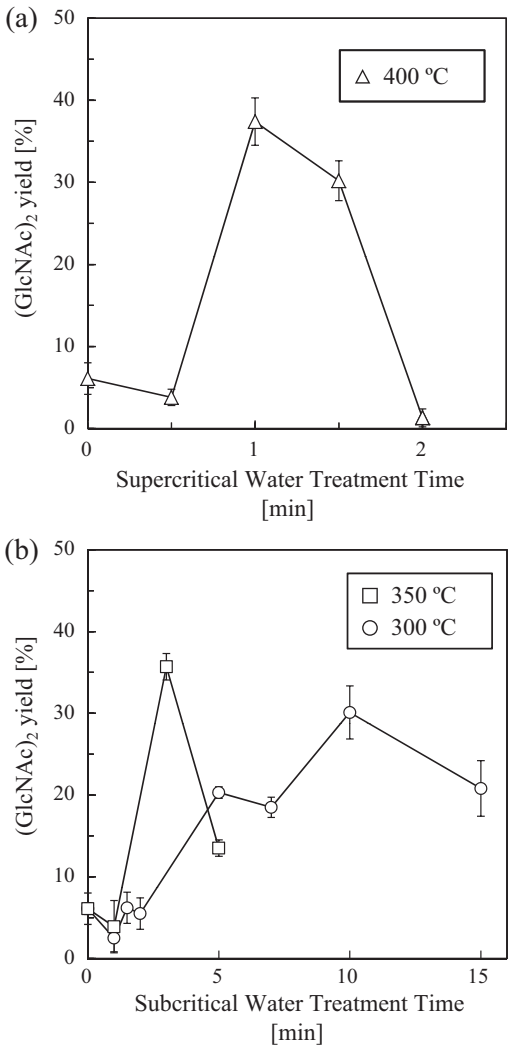

Fig. 3. Effect of (a) supercritical and (b) subcritical water pretreatment time on enzymatic degradation of chitin, using an enzyme reaction time of 48 h ($n = 3$, bars indicate SDs).

Fig. 3 shows the temperature effect of (a) supercritical and (b) subcritical water treatment on the yield of $(GlcNAc)_2$ after enzymatic degradation for 48 h. At 400 °C, the yield of $(GlcNAc)_2$ increased with treatment time and reached 37% at 1 min, after which it decreased to approximately 2% at 2 min. At 350 °C, the peak of $(GlcNAc)_2$ yield was at about 3 min, and at 300 °C, the yield of $(GlcNAc)_2$ peaked at around 10 min. Therefore, the time at which the maximal yield occurred after treatment in sub- and supercritical water increased with decreasing temperature. Interestingly, the maximum yields of $(GlcNAc)_2$ were similar at each temperature, with values in the 30–40% range.

In absence of water, treatment at 400 °C for 1 min resulted in the chitin flacks becoming a black solid resembling char. The yield of $(GlcNAc)_2$ by enzymatic degradation of the black solid was 0%, indicating that the substrate might be denatured. Therefore, these results confirmed the requirement for water in the pretreatment of chitin.

3.2. Properties of the sub- and supercritical water treated chitin

The size of untreated chitin flakes was about 3 mm × 3 mm × 0.5 mm and no significant difference in size was observed until after 1.5 min of supercritical water treatment at 400 °C. After 2 min of supercritical water treatment at 400 °C, the chitin flakes crumbled and became a powder. At 350 and 300 °C, the chitin flakes crumbled after 5 and 15 min of subcritical water treatment, respectively.

The results shown in Fig. 4 were obtained by XRD analysis. Fig. 4(a) and (b) shows the effect of supercritical and subcritical water treatment temperature and time on the crystallinity index of chitin flakes. At 400 °C, the crystallinity index decreased slightly up to 1.5 min and decreased drastically by 2 min. The crystallinity index at 350 °C also decreased slightly up to 3 min and then decreased drastically. In contrast, the crystallinity index was almost unchanged after treatment at 300 °C

Fig. 4(c) and (d) shows the effect of supercritical and subcritical water treatment on the *d*-spacing of chitin flakes. At 400 °C, the *d*-spacing increased markedly during the first 1.5 min, indicating that the lattice spacing became wider. The *d*-spacing then decreased slightly by 2 min. The *d*-spacing at 350 °C also increased during the first 3 min and then decreased by 5 min. At 300 °C, the *d*-spacing gradually increased for the first 5 min but did not change significantly after that time.

Fig. 4(e) and (f) shows the effect of supercritical and subcritical water treatment on the crystallite size of chitin flakes. At 400 °C, the crystallite size increased with treatment time, indicating that the crystal grows in response to supercritical water treatment, however, crystallite size started to decrease at 2 min. The crystallite size also increased up to 3 min at 350 °C, after which it decreased. At 300 °C, the crystallite size gradually increased for the first 5 min but did not change significantly after that time.

Fig. 5 illustrates the FT-IR spectra of chitin before and after the supercritical water treatment. The FT-IR spectra were not changed for the first 1.5 min. At 2 min, the hydrogen bonds in the chitin around 3200–3400 cm^{-1} retracted and the cleavage of the cyclic ether linkage around 1000 cm^{-1} in the glucosamine skeleton unit was enhanced. The ether linkages around 1400 cm^{-1} between the *N*-acetylglucosamine skeleton unit were hydrolyzed at 2 min of supercritical water treatment. The deacetylation reaction of the *N*-acetyl group (C=O at 1700 cm^{-1}) at C-2 of the *N*-acetyl glucosamine unit occurred at 2 min of supercritical water treatment. FT-IR spectroscopy showed that the chitin structure was stable up to 1.5 min at 400 °C.

4. Discussion

The conversion of microcrystalline cellulose to cellooligosaccharides and glucose in supercritical water has been reported to proceed through the following steps (1) swelling, (2) dissolution, and (3) hydrolysis (Sasaki, Adschiri, & Arai, 2004). In this study, we used a batch type reactor, in which the treatment times included heat-up time (Fig. 1). The chitin was stable and was not denatured up to 0.5 min at 400 °C; therefore, the yield of $(GlcNAc)_2$ was not significantly higher than that for untreated chitin. Most chitin swelling occurred between 1.0 and 1.5 min, and the swelling of the chitin was consistent with the shape of the chitin flakes in this time interval. The swollen chitin in supercritical water underwent rapidly cooling. Therefore, the crystallinity index was maintained, although the *d*-spacing increased slightly due to the swelling. The increase of *d*-spacing causes an increase of the crystallite size. After 2 min at 400 °C, dissolution and hydrolysis of the chitin occurred, as indicated by XRD analysis (Fig. 4(a), (c), and (e)). The FT-IR spectra were also changed (Fig. 5), and this was associated with chitin flake crumbling. We analyzed the solution recovered after 2 min of supercritical water treatment; however, $(GlcNAc)_2$ and GlcNAc were not detected, indicating that these compounds immediately decomposed in supercritical water, as reported in literature (Sakanishi et al., 1999; Quitain et al., 2001). At 350 and 300 °C, chitin

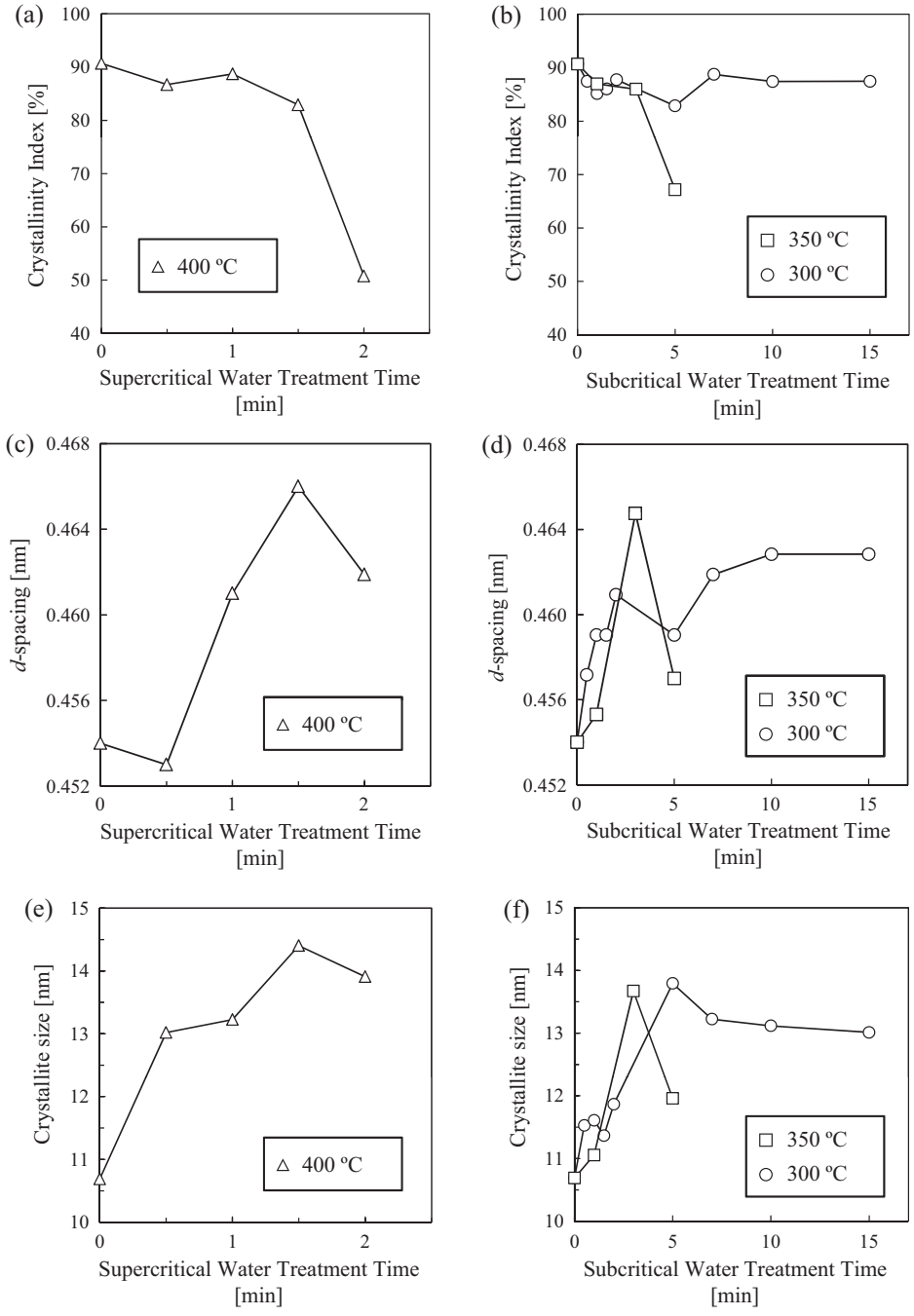

Fig. 4. Effect of sub- and supercritical water pretreatment time on (a-b) crystallinity index, (c-d) *d*-spacing, and (e-f) crystallite size of chitin.

swelling occurred up to 3 and 10 min, respectively, followed by dissolution and hydrolysis of the chitin. These results indicate that there is an optimum pretreatment time at each temperature for chitin swelling without dissolution and hydrolysis.

We previously found that the crystallinity index had a major influence on enzymatic degradation of chitin (Nakagawa et al., 2011). Mechanochemical grinding with a ball mill resulted in a decrease in the crystallinity index of chitin and a corresponding increase in the enzymatic degradation ratio. In this study, the crystallinity index did not change significantly up to 1.5 min (at 400 °C), 3 min (at 350 °C), and 15 min (at 300 °C) as shown in Fig. 4(a) and (b); however, the yield of $(GlcNAc)_2$ showed peaks at earlier treatment times of 1.0 min (at 400 °C), 3 min (at 350 °C), and 10 min (at 300 °C) as shown in Fig. 3. In a separate study, pretreatment of chitin with phosphoric acid or hydrochloric acid at ambient temperature resulted in a high enzymatic degradation ratio, but these pretreatments did not affect the chitin (Ilankovan, Hein, Ng, Trung, & Stevens, 2006). These results indicate that a decrease of the crystallinity index is not essential for promoting the enzymatic degradation of chitin.

The *d*-spacing of the chitin increased after sub- and supercritical water treatment, leading to an increase in crystallite size. This indicated that hydrogen bonds between hydroxyl groups of the chitin chains had become weak and the treated chitin had become swollen. Therefore, there was an increase in the number of the hydroxyl groups without forming hydrogen bonds, which was supported by the gradual decrease in the FT-IR spectra at 3200–3400 cm^{-1} at 1.0 and 1.5 min (Fig. 5). The free hydroxyl

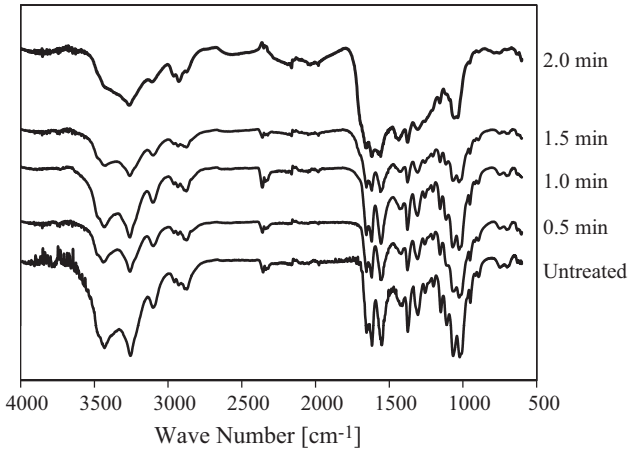

Fig. 5. Effect of supercritical water pretreatment time at 400 °C on FT-IR spectra.

groups would increase the hydrophilicity of the chitin, leading to easier enzyme access to chitin and improved enzymatic degradation.

5. Conclusion

We have demonstrated for the first time that sub- and supercritical water pretreatment leads to significant improvements in the enzymatic degradation of chitin. One minute of supercritical water pretreatment at 400 °C led to an enzymatic degradation ratio of 37%, compared to 5% without the pretreatment. The enzymatic degradation ratio decreased with longer pretreatment times. The same trend was observed in subcritical water at 300 and 350 °C, and the treatment times to achieve the maximum degradation ratio became longer as the treatment temperature decreased. The main product of the enzymatic degradation was $(GlcNAc)_2$. Chitin treated with sub- and supercritical water had a decreased crystallinity index and an increase in *d*-spacing and crystallite size; however, the latter features decreased with longer treatment times. Therefore, it is important to optimize the pretreatment conditions for chitin swelling. Our results indicate that sub- and supercritical water pretreatment followed by enzymatic degradation of chitin is a promising method for reducing the environmental burdens associated with GlcNAc and $(GlcNAc)_2$ production from chitin.

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