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Effects of supercritical water and mechanochemical grinding treatments on physicochemical properties of chitin

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ABSTRACT

This study examined the effects of a combined pretreatment with supercritical water and mechanochemical grinding with a ball mill on the physicochemical properties of chitin and its enzymatic degradation. Following pretreatment with a combination of supercritical water and grinding, chitin had a lower mean molecular weight, a lower crystallinity index, a lower crystallite size, greater *d*-spacing, weaker hydrogen bonds, and the amide group was more exposed compared with untreated chitin. These properties increased the hydrophilicity of the chitin and enhanced its enzymatic degradation. The *N*,*N*′-diacetylchitobiose $(GlcNAc)_2$ yield after enzymatic degradation of chitin following pretreatment with supercritical water (400 °C, 1 min) and grinding (800 rpm, 10 min) was 93%, compared with 5% without any treatment, 37% with supercritical water pretreatment alone (400 °C, 1 min), and 60% with grinding alone (800 rpm, 30 min).

1. Introduction

N,*N*′-diacetylchitobiose, $(GlcNAc)_2$, is a dimer of *N*-acetylglucosamine (GlcNAc). GlcNAc, derived from crustacean chitin (α-chitin), is a versatile functional compound used in skin moisturizers, analgesics for joint pain, and antitumoral and antimicrobial agents (Liang, Chen, Yen, & Wang, 2007; Liu et al., 2011; Muzzarelli, 2011; Muzzarelli et al., 2012; Suzuki et al., 1986; Wang et al., 2008). $(GlcNAc)_2$ has superior physiological activities compared with GlcNAc and it is an attractive building block for the production of oligomers (Usui, Matsui, & Isobe, 1990). Chitin oligomers have elicitor activities in plants and are implicated in the activation of immune responses, the control of intentional inflammation and the stimulation of bifidobacteria growth (Hirano, 2004). The production of $(GlcNAc)_2$ and GlcNAc from crab shells involves numerous steps that require strongly acidic conditions because of the crystallinity and insolubility of α-chitin. An enzymatic process that depolymerizes chitin completely would be environmentally favorable 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 because of its hydrophobicity, high crystallinity, and strong hydrogen bonds. These properties make the enzymatic depolymerization of α-chitin difficult. In light of this, pretreatments that reduce the hydrophobicity of chitin, by loosening the crystal structure and weakening the hydrogen bonds, are important.

Previously, we reported that sub- and supercritical water (Tc = 374.3 °C, Pc = 22.1 MPa) pretreatment improves the enzymatic degradation of chitin and the $(GlcNAc)_2$ yield after enzymatic degradation with an optimum supercritical water pretreatment at 400 °C for 1.0 min was 37%, compared to 5% without pretreatment (Osada et al., 2012). We also reported that mechanochemical grinding pretreatment of chitin using a ball mill enhanced enzymatic degradation (Nakagawa et al., 2011). Although each pretreatment with supercritical water and mechanochemical grinding was effective for enzymatic degradation, detailed physicochemical properties of chitin after these pretreatments were not investigated. In addition, combined pretreatment with supercritical water and mechanochemical grinding of chitin was not conducted. The aim of this study was to investigate the effect of the combined pretreatment with supercritical water and mechanochemical grinding on physicochemical properties of chitin and its enzymatic degradation.

2. Materials and methods

2.1. Materials and enzymes

The sources of chitin and enzymes have been reported previously (Osada et al., 2012).

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2.2. Supercritical water pretreatment

The method used for the pretreatment of chitin with supercritical water was reported previously (Osada et al., 2012). The treatment conditions used in this study were 400 °C for 1.0 min.

2.3. Mechanochemical grinding pretreatment

The grinding equipment was a converge mill produced by Makabe Giken (internal capacity, 1000 cm^3). The details of the mill structure were reported previously (Nakagawa et al., 2011). Two grams of the chitin and 704 g of chromium steel balls (5 mm dia.) were ground at 800 rpm for 10–30 min.

2.4. Average particle size

The average particle size (median size D_{50}) was determined using a particle size distributor (Nikkiso, HRA [X-100]). Methanol was used to disperse the samples.

2.5. Surface area

The Brunauer–Emmett–Teller (BET) surface areas of untreated and pretreated chitins were determined using a nitrogen adsorption method (Bel Japan, Belsorp-2).

2.6. Molecular weight distribution

The molecular weight distribution of chitin was measured using a gel permeation chromatography (GPC) system (Shimadzu, LC-10Avp), which was equipped with a refractive index detector, a GPC column (Tosoh, TSK gel G5000H_{HR}), and a guard column (Tosoh, TSK guard column HHR-H). Pullulan standards (Shodex STANDARD P-82) of 708, 340, 200, 107, 47.1, 21.1, 9.6, and 5.9 kDa were used. A total of 1% of the sample was dissolved in 5% LiCl/DMAC with continuous stirring for two days. Prior to measurement, the samples were filtered through a 0.45 μm nylon filter. The flow rate of the mobile phase was 0.5 mL min^{-1}. The temperatures of the columns were set at 30 °C and the detector was 30 °C. A calibration curve was plotted for the elution time versus the absolute molecular weight of the standards. The relative mean molecular weight of the chitin samples was estimated from the standard curve.

2.7. X-ray diffraction (XRD)

The method used to calculate the crystallinity index of the (1 1 0) lattice (at $2\theta = 20$), the *d*-spacings of the (1 1 0) and (0 2 0) (at $2\theta = 9$) lattices, and the crystallite size of the (1 1 0) lattice from the XRD data were identical to those described previously (Osada et al., 2012).

2.8. Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of chitin were measured using a Nicolet iS10 spectrometer (Thermo Fisher Scientific Inc.).

2.9. Near-infrared (NIR) spectroscopy

The NIR spectra of chitin were measured using a PlaScan-W spectrometer (OPT Research Inc.).

2.10. Thermogravimetric and differential thermal analysis (TG-DTA)

Thermal analysis of chitin was conducted in a nitrogen atmosphere using a TG-DTA instrument (Rigaku, Thermo plus EVO TG-8120). The temperature program was set to produce a temperature range of 30–600 °C at a rate of 20 °C min^{-1}.

2.11. Enzymatic degradation of chitin

The conditions used for the enzymatic degradation of chitin were reported previously (Osada et al., 2012).

2.12. High-performance liquid chromatography (HPLC)

The HPLC system and the method used to calculate the yields of $(GlcNAc)_2$ and GlcNAc have been reported previously (Osada et al., 2012).

3. Results

3.1. Average particle size

Table 1 shows the effect of various pretreatments on the average particle diameter, as well as mean molecular weight, crystallinity index, *d*-spacing of the (1 1 0) and (0 2 0) lattices, the crystallite size, and the $(GlcNAc)_2$ yield after enzymatic degradation for 48 h.

The average particle size after supercritical water pretreatment was approximately 3000 μm (run 2), which was the same as that of the untreated chitin flakes (run 1). After grinding both with and without supercritical water pretreatment, the particle size decreased significantly to 13–24 μm (runs 3–8). The particle size after combined pretreatment with supercritical water and grinding for 10 min was 18 μm (run 3), which was slightly larger than that observed when grinding was performed for 30 min (run 4). When grinding was performed alone for 10 min or 30 min, the particle size decreased with increasing grinding time (runs 5 and 6). After combined pretreatment of grinding for 10 min or 30 min followed by supercritical water treatment, the particle size was 24 μm in both cases (runs 7 and 8), which was almost the same size as when only grinding was performed (runs 5 and 6). When grinding was performed after supercritical water treatment (runs 3 and 4), the particle size was smaller than that observed with grinding alone (runs 5 and 6). These results indicate that supercritical water pretreatment made the chitin flakes fragile.

3.2. Surface area

The BET surface areas during runs 2–8 were from 22–36 $m^2\ g^{-1}$, which were similar to that of untreated chitin flakes (33 $m^2\ g^{-1}$). These results indicate that the supercritical water and grinding pretreatment did not affect the surface area, although the particle diameters after grinding (runs 3–8) were lower than those before grinding (runs 1 and 2) as shown in Table 1.

3.3. Mean molecular weight

The untreated chitin flake was not dissolved completely in the LiCl/DMAC solvent and we measured the mean molecular weight of only soluble part of chitin. Therefore, the real mean molecular weight of untreated chitin would be higher than 760 kDa. On the other hand, the chitin samples of runs 2–8 were dissolved completely in the LiCl/DMAC solvent. The mean molecular weight decreased to 209 kDa following supercritical water pretreatment (run 2). Following combined pretreatment with supercritical water and grinding for 10 and 30 min (runs 3 and 4), the mean molecular weight decreased significantly. The mean molecular weight was also reduced after grinding alone for either 10 or 30 min (runs 5 and 6), but the reduction was greater when both treatments were performed (runs 3 and 4). This result indicates that the grinding pretreatment reduced the mean molecular weight by making the

Table 1
Effects of supercritical water and mechanochemical grinding pretreatments on the properties of chitin and its enzymatic degradation.

Run		Particle diameter (μm)	Mean molecular weight (kDa)	Crystallinity index (1 1 0) (%)	*d*-spacing (1 1 0) (nm)	*d*-spacing (0 2 0) (nm)	Crystallite size (1 1 0) (nm)	$(GlcNAc)_2$ yield (%)
1	Untreated	3000	>760	91	0.454	0.921	10.7	5 ± 1
2	Supercritical water treatment (400 °C, 1 min)	3000	209	88	0.461	0.952	13.2	37 ± 3
3	Supercritical water treatment (400 °C, 1 min) + grinding (10 min)	18	9.5	26	0.458	0.965	2.0	93 ± 4
4	Supercritical water treatment (400 °C, 1 min) + grinding (30 min)	13	4.8	27	0.460	0.977	1.8	65 ± 2
5	Grinding (10 min)	24	407	68	0.462	0.965	8.8	40 ± 8
6	Grinding (30 min)	22	267	40	0.460	0.977	2.7	60 ± 6
7	Grinding (10 min) + supercritical water treatment (400 °C, 1 min)	24	5.2	78	0.464	0.952	14.7	23 ± 3
8	Grinding (30 min) + supercritical water treatment (400 °C, 1 min)	24	4.6	71	0.464	0.964	12.1	55 ± 5

supercritical water easier. However, the mean molecular weights were also reduced after grinding for 10 or 30 min followed by supercritical water treatment (runs 7 and 8). The low molecular weights in runs 7 and 8 are explained in Section 4.4.

3.4. Crystallinity index

The crystallinity index after the supercritical water pretreatment (run 2) was slightly lower than that of the untreated chitin flakes (run 1). After the combined pretreatment with supercritical water and grinding for 10 and 30 min, the crystallinity index decreased significantly to 26 and 27%, respectively (runs 3 and 4). Following grinding for 10 or 30 min, the crystallinity indices decreased gradually with increasing grinding time (runs 5 and 6), but they remained higher than when supercritical water treatment was performed before grinding (runs 3 and 4). After grinding for 10 or 30 min followed by supercritical water treatment (runs 7 and 8), the crystallinity index was higher than with grinding alone for 10 or 30 min (runs 5 and 6). Interestingly, the lower crystallinity indices after the initial grinding (runs 5 and 6) were increased by the subsequent supercritical water pretreatment (runs 7 and 8), indicating that recrystallization of the chitin crystal structure occurred in supercritical water. The reduced crystallinity indices observed when supercritical water pretreatment was combined with grinding (runs 3 and 4) were achieved with shorter grinding times than when supercritical water pretreatment was omitted (runs 5 and 6). These results indicate that the chitin crystal structure was easier to break following supercritical water pretreatment. The crystallinity indices with supercritical water and grinding for 10 or 30 min (runs 3 and 4) were similar, indicating that the chitin crystal structure was broken within 10 min of grinding.

3.5. d-spacing

The *d*-spacing of the (1 1 0) lattice after supercritical water and/or grinding pretreatment (runs 2–8) was greater than that of untreated chitin flakes (run 1). We checked the reproducibility of the *d*-spacing values and the error was ±0.03 nm. This indicates that the difference in the *d*-spacing values of the (1 1 0) lattice in runs 2–8 was not significant. The *d*-spacing of the (0 2 0) lattice was affected more by the pretreatment than that of the (1 1 0) lattice. The *d*-spacing of the (0 2 0) lattice after the supercritical water pretreatment (run 2) was greater than that of untreated chitin flakes (run 1). When supercritical water treatment was followed by grinding, the *d*-spacing of the (0 2 0) lattice increased with the grinding time (runs 3 and 4). When grinding was performed alone, the *d*-spacing of the (0 2 0) lattice increased with increasing grinding time (runs 5 and 6). Interestingly, the *d*-spacing of the (0 2 0) lattice after combined pretreatment with grinding followed by supercritical water (runs 7 and 8) was lower than that with grinding alone (runs 5 and 6). The cause of the smaller *d*-spacing of the (0 2 0) lattice of runs 7 and 8 compared with runs 5 and 6 will be discussed later. The *d*-spacing of the (1 1 0) and (0 2 0) lattices indicates that the distance between adjacent chitin chains was increased by the supercritical water and grinding pretreatments.

3.6. Crystallite size

The crystallite size after supercritical water pretreatment alone (run 2) was slightly larger than that of untreated chitin flakes (run 1). The crystallite size decreased significantly after the combined pretreatment with supercritical water followed by grinding for 10 or 30 min (runs 3 and 4). The crystallite size after grinding alone decreased with increasing grinding time (runs 5 and 6). Interestingly, the crystallite sizes after grinding for 10 or 30 min followed by supercritical water (runs 7 and 8) were larger than those with grinding alone (runs 5 and 6). This indicates that the crystallite sizes of the untreated chitin (run 1) were reduced by grinding (runs 5 and 6), but that they were increased by the supercritical water pretreatment (runs 7 and 8). This was likely due to the swelling of chitin in supercritical water and the growth of the crystal structure. These phenomena also occurred from run 1 to run 2.

3.7. FTIR

Fig. 1 shows the FTIR spectra of chitin before and after the supercritical water and grinding treatments (runs 1–8). The shapes of the peaks around 800–1700 cm^{-1} during runs 2–8 were similar to those in run 1, indicating that the chitin structure was retained after all the pretreatments. The FTIR spectrum around 3200–3500 cm^{-1} after supercritical water pretreatment (run 2) was similar to that of untreated chitin flakes (run 1). The chitin peaks around 3200–3500 cm^{-1} after grinding pretreatments (runs 3–8) differed from those of unground chitin (runs 1 and 2). The peaks around 3200–3500 cm^{-1} in runs 3 and 4 were smaller and smoother than those in runs 5–8. Sikorski, Hori, and Wada (2009) reported that there are three hydrogen bonds (O—H stretching) between adjacent chitin chain (3268 cm^{-1}) and two between chitin

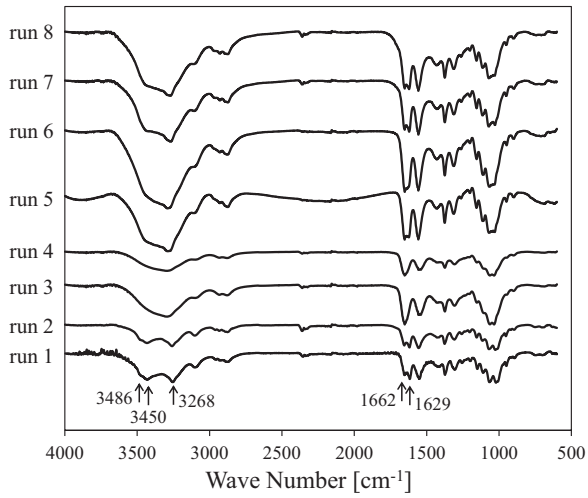

Fig. 1. Effects of supercritical water and mechanochemical grinding pretreatments on FTIR spectra.

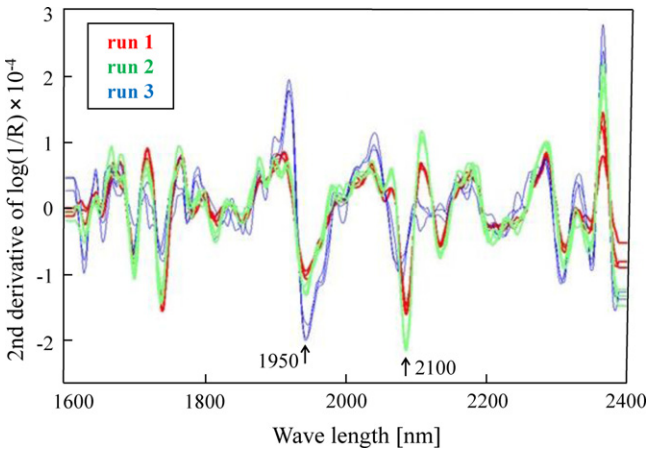

Fig. 2. Effects of supercritical water and mechanochemical grinding pretreatments on NIR spectra.

interchains (3450 and 3486 cm^{-1}). The latter two interchain hydrogen bonds were weakened by grinding (runs 3–8). By contrast, the hydrogen bonds between the adjacent chitin chains at 3268 cm^{-1} were retained in runs 5–8. In runs 3 and 4, the three peaks at 3268, 3450, and 3486 cm^{-1} became smaller and smoother than those in runs 5–8, indicating that the weakening of the three hydrogen bonds in runs 3 and 4 occurred more readily than in runs 5–8. Furthermore, the peak at 1629 cm^{-1} was larger than that at 1662 cm^{-1} in runs 1, 2, 7, and 8, whereas the opposite was observed in runs 5 and 6. In runs 3 and 4, the two bands formed a single peak. The peaks at 1629 and 1662 cm^{-1} were attributed to two types of hydrogen bonds in a C=O group with the NH group of the adjacent chain and the OH group of the interchain (Wu, Sasaki, Irie, & Sakurai, 2008). The hydrogen bond between the C=O group and the OH group of the interchain (1662 cm^{-1}) was in the same direction as the two hydrogen bonds between OH groups (3450 and 3486 cm^{-1}), which were weakened by the grinding treatment. The hydrogen bond between the C=O group and the NH group in the adjacent chains (1629 cm^{-1}) was also in the same direction as the hydrogen bonds between OH groups (3268 cm^{-1}) and the *d*-spacing of the (1 1 0) lattice. The change in the FTIR spectra around 1629 and 3268 cm^{-1} was not clear, but the *d*-spacing of the (1 1 0) lattice was larger with supercritical water and grinding pretreatment, as mentioned in Section 3.5.

3.8. NIR

Fig. 2 shows the NIR spectra of chitin after no pretreatment (run 1), supercritical water pretreatment (run 2), and combined supercritical water and grinding pretreatment (run 3). The peak size of the amide group around 2100 nm was in the order of: runs 3 < 1 < 2, indicating that the amide group was exposed by the supercritical water pretreatment. The peak size around 1950 nm, which was assigned to the hydroxyl group, was in the order of: runs 1 < 2 < 3. It has been reported that the peak around 1950 nm increases with water content of the chitin crystal structure (García Mir et al., 2011). These results indicate that the chitin crystal structure contained more water following combined supercritical water and grinding pretreatment. All of the chitin samples were dried at 90 °C for 24 h before NIR analysis. However, since the samples were exposed to the atmosphere during the NIR measurements, it is possible that they may have absorbed moisture.

3.9. TG-DTA

Fig. 3 shows the TG and DTA curves of chitin for runs 1–3. The TG and DTA results in run 1 were similar to those of run 2. The peak around 400 °C for run 2 with DTA was slightly higher than that of run 1 (Fig. 3b); but several repeats of the DTA measurements using the same sample confirmed that this difference was not significant. The TG curve of run 3 fell twice at around 100 and 300 °C (Fig. 3a), while the DTA curve of run 3 had two peaks at around 100 and 300 °C (Fig. 3b). The DTA peak in run 3 at around 100 °C was due to the evaporation of water, although all samples were pre-dried at

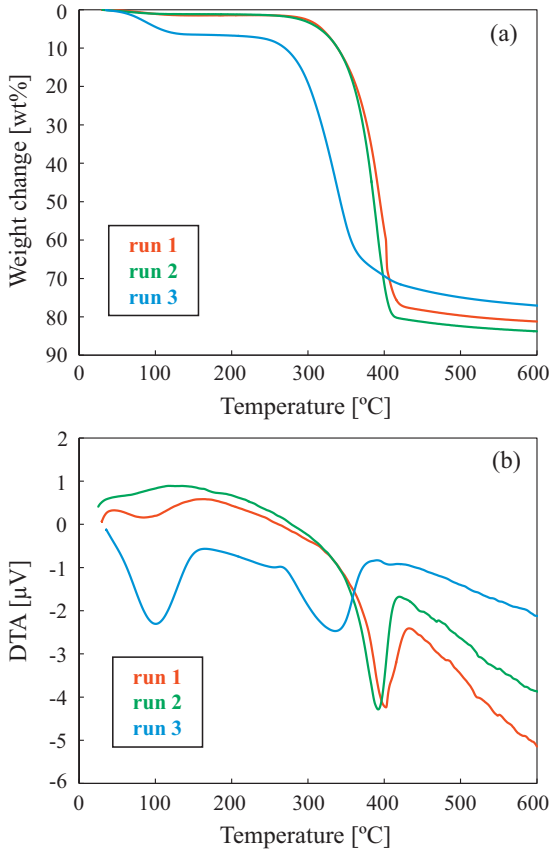

Fig. 3. Effects of supercritical water and mechanochemical grinding pretreatments on (a) TG and (b) DTA curves.

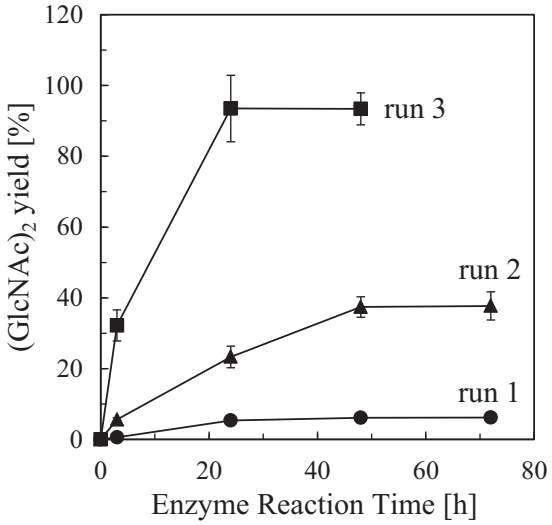

Fig. 4. Reaction time profile of the enzymatic degradation of untreated chitin (run 1), chitin pretreated with supercritical water (run 2), and chitin pretreated with a combination of supercritical water and mechanochemical grinding (10 min) (run 3) ($n = 3$, bars indicate the SDs).

90 °C for 24 h. The decrease in TG and the DTA peak around 300 °C in run 3 was due to the pyrolytic decomposition of chitin, which was significantly less than that in runs 1 and 2. These results indicate that the pyrolysis of chitin following combined supercritical water and grinding pretreatment (run 3) occurred at a lower temperature compared with untreated chitin (run 1) and chitin treated with supercritical water (run 2). The pyrolysis temperature of polymers is reduced as the mean molecular weight decrease (Qin et al., 2004). The lower pyrolysis temperature in run 3 compared with runs 1 and 2 corresponded to the lower molecular weight in run 3 compared with that in runs 1 and 2 (Table 1).

3.10. Enzymatic degradation of chitin

Fig. 4 shows the $(GlcNAc)_2$ yield after enzymatic degradation with no pretreatment (run 1), supercritical water pretreatment (run2), and a combination of supercritical water and grinding pretreatment (10 min) (run3). With no pretreatment (run 1), the $(GlcNAc)_2$ yield at 72 h was 5%. The $(GlcNAc)_2$ yield after pretreatment with supercritical water was 37% at 72 h (run 2). Our previous study showed that the optimum pretreatment condition was supercritical water at 400 °C for 1 min (Osada et al., 2012). When supercritical water treatment was followed by grinding for 10 min (run 3), the $(GlcNAc)_2$ yield increased significantly to 93% at 24 h, indicating that most of the chitin was converted to $(GlcNAc)_2$. Fig. 4 shows that enzymatic degradation was complete by 48 h. By contrast, the GlcNAc yield after enzymatic degradation for 72 h in runs 1–3 increased by only 2% (data not shown); therefore, we focused our analysis on $(GlcNAc)_2$. We also analyzed the aqueous solution that was recovered after supercritical water treatment without subsequent enzymatic degradation, but $(GlcNAc)_2$ and GlcNAc were not detected.

The $(GlcNAc)_2$ yields after 48 h of enzymatic degradation in runs 1–3 were 5%, 37%, and 93%, respectively (Table 1 and Fig. 4). The use of a combination of supercritical water followed by a longer grinding pretreatment time of 30 min decreased the $(GlcNAc)_2$ yield to 65% (run 4). With grinding pretreatment alone for 10 or 30 min, the $(GlcNAc)_2$ yields were 40% and 60%, respectively (runs 5 and 6). The $(GlcNAc)_2$ yield reached a maximum at 30 min of grinding pretreatment, after which the yield began to decrease with increasing grinding time. The order of pretreatment with supercritical water and grinding was then switched. Grinding for 10 min followed by supercritical water generated a $(GlcNAc)_2$ yield of 23% (run 7), which was lower than that with grinding alone for 10 min (run 5). A combination of grinding for 30 min followed by supercritical water produced a $(GlcNAc)_2$ yield of 55% (run 8), which was also lower than that with grinding alone for 30 min (run 6). These results indicate that initial pretreatment with supercritical water followed by grinding enhanced the enzymatic degradation of chitin. The optimum grinding period following supercritical water pretreatment was 10 min, whereas if supercritical water pretreatment was not performed, 30 min of grinding was optimal.

4. Discussion

4.1. Effects of supercritical water pretreatment

Our recent study showed that the reaction of chitin in supercritical water proceeds via three steps: (1) swelling, (2) dissolution, and (3) hydrolysis, with increasing pretreatment time (Osada et al., 2012). The supercritical water pretreatment applied in this study (400 °C for 1.0 min) was in the middle of the swelling step (1), so the particle diameter and the crystallinity index were retained. However, the *d*-spacing of the (1 1 0) lattice and the crystallite size increased slightly due to swelling while the hydrogen bonds were weakened, according to the FTIR spectra. The current study showed the followings: (1) The mean molecular weight of chitin was decreased after supercritical water pretreatment, indicating slight hydrolysis during the chitin swelling step. (2) The crystallinity index and the crystallite size were reduced by the initial grinding pretreatment (runs 5 and 6) but they increased again after the supercritical water pretreatment (runs 7 and 8). This indicated that the amorphous chitin structure was recrystallized and growth of the crystal structure resumed during supercritical water pretreatment. (3) An increased number of exposed amide groups was detected in the NIR spectra, which were attributable to the increased *d*-spacings, i.e., the distance between chitin chains due to weakened hydrogen bonds. Therefore, the effects of the supercritical water pretreatment on chitin were follows: (A) a decrease in the mean molecular weight, (B) growth of the crystallite structure, and (C) an increase in the distance between chitin chains. While (A) and (C) would assist the enzymatic degradation of chitin, (B) would hinder degradation. Thus, the $(GlcNAc)_2$ yield in run 2 was due to the positive and negative effects of supercritical water pretreatment described here.

4.2. Effects of mechanochemical grinding pretreatment

It was previously reported that mechanochemical grinding pretreatment reduced the particle size and the crystallinity index of chitin (Nakagawa et al., 2011). The current study showed the followings: (1) The surface area after grinding was similar to that observed with no pretreatment, although grinding did decrease particle size. (2) The mean molecular weight was reduced by the grinding pretreatment. (3) The *d*-spacings of the (1 1 0) and (0 2 0) lattices were increased by grinding. (4) The interchain hydrogen bonds were weakened following grinding, according to the FTIR spectra. (5) The crystallite size was decreased after grinding. Therefore, the effects of the grinding pretreatment, i.e., the mechanochemical effect, on chitin were follows: (A) a decrease of the mean molecular weight: and (B) the amorphization of the crystallite structure, which involved an increase in the distance between adjacent chitin chains, a weakening of the interchain hydrogen bond, and a decrease in the crystallite size. (A) and (B) have positive effects on the enzymatic degradation of chitin. However, increasing the grinding to more than 30 min had a

negative effect on the $(GlcNAc)_2$ yield, indicating that a decrease in the molecular weight and breakage of the chitin chemical structure occurs after prolonged grinding pretreatment. After 30 min of grinding, a proportion of the chitin still retained a high molecular weight and high crystallinity. This explain why the $(GlcNAc)_2$ yield after grinding reached only a maximum 60%.

4.3. Effects of the combined pretreatment with supercritical water and grinding

The mean molecular weight in run 3 was significantly lower than that in run 5, although both runs included 10 min grinding pretreatment. The *d*-spacing of the (1 1 0) and (0 2 0) lattices was increased by the supercritical water pretreatment (run 2). Therefore, grinding was more effective after the supercritical water pretreatment (runs 3 and 4) and decreased the mean molecular weight, the crystallinity index, and the crystallite size compared to chitin with no pretreatment (runs 5 and 6). This was reflected in the smoother FTIR spectra in runs 3 and 4 compared with runs 5 and 6. The $(GlcNAc)_2$ yield in run 4 was lower than that in run 3 while the FTIR spectra in run 4 was smoother than that in run 3, which indicate that partial decomposition of the chitin chemical structure occurred after grinding for 30 min. The NIR and TG-DTA results indicate that chitin absorbed moisture from the air more easily in run 3 compared with runs 1 and 2. This is likely because more OH groups were exposed after breaking the chitin crystal structure in run 3 compared with runs 1 and 2. The high hydrophilicity of the chitin of run 3 most likely explains why it was more easily accessible to the enzyme and more susceptible to degradation.

4.4. Effects of the order of pretreatment with supercritical water and mechanochemical grinding

Table 1 shows that the $(GlcNAc)_2$ yields in runs 7 and 8 were lower than those in runs 5 and 6, indicating that grinding followed by supercritical water pretreatment had a negative effect on the enzymatic degradation of chitin. The particle diameter in runs 7 and 8 was similar to that in runs 5 and 6. The FTIR peaks at 3450 and 3486 cm^{-1} in runs 7 and 8 were slightly higher than those in runs 5 and 6, which indicate that the hydrogen bonds between the interchain OH groups in runs 7 and 8 were stronger than those in runs 5 and 6. As mentioned in Section 4.1, the supercritical water pretreatment promoted the growth and recrystallization of the chitin crystal structure. The *d*-spacing of the (0 2 0) lattice in runs 7 and 8 was smaller than that in runs 5 and 6, which indicates that recrystallization decreased the distance between adjacent chitin chains. However, the *d*-spacing of the (0 2 0) lattice in runs 7 and 8 was still larger than that in run 1. The higher crystallinity index and crystallite size in runs 7 and 8 compared with run 5 and 6 lead to a lower $(GlcNAc)_2$ yield after enzymatic degradation. The mean molecular weight in runs 7 and 8 was significantly lower than that in run 2, which indicates that the hydrolysis of chitin by supercritical water was more effective in runs 7 and 8 compared with run 2. This was probably due to the lower mean molecular weight before the supercritical water pretreatment in runs 5 and 6 compared with run 1. Following the grinding pretreatment chitin (runs 5 and 6), chitin was more readily hydrolyzed by supercritical water and decomposition of the chitin chemical structure occurred within 1 min of the supercritical water pretreatment.

5. Conclusions

For the first time, this study demonstrates that a combined supercritical water and mechanochemical grinding pretreatment significantly affects the physicochemical properties of chitin and enhances the enzymatic degradation of chitin. The supercritical water pretreatment decreased the mean molecular weight and increased the distance between chitin chains by weakening hydrogen bonds. The grinding pretreatment caused an additional decrease in the mean molecular weight and amorphization of the crystallite structure. These effects made the chitin more hydrophilic, which promoted enzymatic degradation. A 1 min supercritical water treatment at 400 °C followed by 10 min mechanochemical grinding produced a $(GlcNAc)_2$ yield of 93%, compared with 5% without any pretreatment. This combined pretreatment was more effective at enhancing the enzymatic degradation of chitin, than when each pretreatment was performed separately. The current study showed that a combined pretreatment of chitin with supercritical water and mechanochemical grinding followed by enzymatic degradation is a promising method for reducing the environmental burdens associated with producing GlcNAc and $(GlcNAc)_2$ from chitin.

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