Effect of Sub- and Supercritical Water Treatments on the Physicochemical Properties of Crab Shell Chitin and its Enzymatic Degradation

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Abstract

This study examined the effects of sub- and supercritical water pretreatments on the physicochemical properties of crab shell α-chitin and its enzymatic degradation to obtain *N*,*N*'-diacetylchitobiose $(GlcNAc)_2$. Following sub- and supercritical water pretreatments, the protein in the crab shell was removed and the residue of crab shell contained α-chitin and $CaCO_3$. Prolonged pretreatment led to α-chitin decomposition. The reaction of pure α-chitin in sub- and supercritical water pretreatments was investigated separately; we observed lower mean molecular weight and weaker hydrogen bonds compared with untreated α-chitin. $(GlcNAc)_2$ yields from enzymatic degradation of subcritical (350°C, 7 min) and supercritical water (400°C, 2.5 min) pretreated crab shell were 8% and 6%, compared with 0% without any pretreatment. This study shows that sub- and supercritical water pretreatments of crab shell provide to an alternative method to the use of acid and base for decalcification and deproteinization of crab shell required for $(GlcNAc)_2$ production.

Keywords: Chitin; Enzymatic degradation; Marine biomass; *N*-acetyl glucosamine; Hydrothermal treatment

1. Introduction

Chitin is the second most abundant biomass on earth after cellulose and is a major component of the cell walls of fungi as well as of the exoskeletons of insects and crustaceans (Ravi Kumar et al., 2004). *N*,*N*'-diacetylchitobiose, $(GlcNAc)_2$, is a dimer of *N*-acetyl glucosamine (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 (Muzzarelli, 2011; Muzzarelli et al., 2012). $(GlcNAc)_2$ is also a useful product, acting as an inducer in the production of chitinolytic enzymes (Uchiyama et al., 2003). Moreover, it is a suitable building block for the production of chitin oligomers by enzymatic transglycosylation (Usui et al., 1990). Chitin oligomers have elicitor activities in plants and have been implicated in the activation of immune responses, the regulation of intentional inflammation, and the stimulation of bifidobacteria growth (Aam et al., 2010; Hirano, 2004). The production of α-chitin from crab shells involves numerous steps that require a strong acid and a base to remove $CaCO_3$ and protein in the crab shell. If the direct conversion of crab shell to $(GlcNAc)_2$ can be achieved using enzymes, it would not be necessary to use deleterious substances or to produce an excessive amounts of waste water, thereby allowing for a more environmentally friendly process.

Sub- and supercritical water (Tc = 374.3°C, Pc = 22.1 MPa) have been recognized as a green chemical medium for some organic reactions that can proceed without any catalyst.

Previously, we have reported that sub- and supercritical water pretreatments improve enzymatic degradation of pure α-chitin (Osada et al., 2012). The $(GlcNAc)_2$ yield after enzymatic degradation with optimum supercritical water pretreatment at 400°C for 1.0 min was about 7 times greater than that without pretreatment. Although sub- and supercritical water pretreatments were effective for enzymatic degradation of pure α-chitin, the effect of these pretreatments on crab shell has not been investigated.

The effect of sub- and supercritical water treatments on α-chitin present in biomass such as crab shell has been reported. Quitain et al. (2001) treated shrimp shell in sub- and supercritical water up to 400°C, and obtained amino acids through the hydrolysis of proteins in the shrimp shell. However, they could not obtain glucosamine from shrimp shell and claimed that glucosamine might decompose in sub- and supercritical water. Nakamura et al. (2007) treated crab shell in subcritical water up to 350°C, and reported that protein was removed at temperature ranging from 260°C to 320°C, after 1 to 20 min of treatment. In subcritical water, the decomposition of α-chitin was also promoted; however, GlcNAc and glucosamine were not obtained as products. On the other hand, they reported that $CaCO_3$ was stable up to 350°C. Some reports suggest that sub- and supercritical water treatments on their own are not sufficient to obtain $(GlcNAc)_2$ and GlcNAc from crab shell, because under these conditions, $(GlcNAc)_2$ and GlcNAc decompose at the same time that the α-chitin is hydrolyzed (Aida et al., 2014; Sakanishi et al., 1999).

In this study, we used pretreatment of crab shell in sub- and supercritical water for enzymatic degradation and investigated the conditions that promote enzymatic degradation to obtain $(GlcNAc)_2$ directly. We have previously reported the effect of sub- and supercritical water pretreatments on the properties of pure α-chitin; in this study, we have analyzed new data to obtain deeper insights into the structural changes in α-chitin. We will discuss the effect of sub- and supercritical water pretreatments on crab shell properties in comparison with those of pure α-chitin.

2. Materials and Methods

2.1. Materials and enzymes

Crab shell and pure α-chitin were obtained from Yaizu Suisankagaku Industry Co. Ltd. The flake size of the crab shell or pure α-chitin was approximately 3 mm × 3 mm × 0.5 mm. The pure α-chitin was obtained from crab shell by acid and base treatments. The source of enzymes has been reported previously (Osada et al., 2012).

2.2. Sub- and supercritical water pretreatments

The method used for the pretreatment of crab shell or pure α-chitin with sub- and supercritical water has been reported previously (Osada et al., 2012). Crab shell or pure α-chitin (0.2 g) and water (3 g) were loaded in the reactor. The treatment conditions used in

this study were 400°C, 350°C, and 300°C for 0.5–40 min. After sub- and supercritical water pretreatments, the crab shell or pure α-chitin was dried at 90°C for 24 h.

2.3. Weight change

We evaluated the weight change of the crab shell or pure α-chitin after sub- and supercritical water treatments as given below:

$$\text{Weight change (\%)} = \frac{\text{Weight of crab shell (or α-chitin) recovered (g)}}{\text{Weight of crab shell (or α-chitin) loaded (0.2 g)}} \times 100 \quad (1)$$

2.4. X-ray diffraction (XRD)

Equatorial diffraction profiles of crab shell 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.

2.5. Fourier transform infrared (FTIR) spectroscopy

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

2.6. Molecular weight distribution

The molecular weight distribution of pure α-chitin was measured using a gel

permeation chromatography (GPC) system. The details of the GPC analysis have been reported previously (Osada et al., 2013b).

2.7. Thermo gravimetric analysis (TG)

Thermal analysis of pure α-chitin was conducted in a nitrogen atmosphere using a TG 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}.

2.8. Laser scanning microscopy

The surface micrograph and roughness patterns of pure α-chitin were measured using a 3D confocal laser scanning microscope (Keyence Corp., VK-9710). The magnification of the objective lens was set to 50 times.

2.9. Enzymatic degradation of crab shell or pure α-chitin

Enzymatic degradation of crab shell (or pure α-chitin) was conducted using the following protocol. We mixed 20 mg of crab shell (or pure α-chitin) before and after sub- and supercritical water pretreatments (1% of final concentration) with 1.8 mL of 10 mmol/L phosphate buffer (at 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.

2.10. High-performance liquid chromatography (HPLC)

The HPLC system and the method used have been reported previously (Osada et al., 2012). The product yield was defined as given below:

$$\text{Product yield (\%)} = \frac{\text{Weight of } (\text{GlcNAc})_2 \text{ or GlcNAc (g)}}{\text{Weight of crab shell (or α-chitin) loaded (0.2 g)}} \times 100 \quad (2)$$

The definition of product yield is different from our previous research (Osada et al., 2012, 2013b) because the exact amount of α-chitin in the crab shell residue after sub- and supercritical water treatments was not clear. In this study, the denominator of eq. (2) was the weight before sub- and supercritical water treatments and therefore, it was fixed at 0.2 g. On the other hand, in our previous research, the denominator was the weight after sub- and supercritical water treatments and therefore, it changed with the treatment, as in eq. (1). Consequently, the $(GlcNAc)_2$ yield from pure α-chitin in this study was lower than yield reported earlier. Some experiments were repeated three times to confirm reproducibility. The averages of these experiments were shown in the Figs along with the standard errors.

3. Results

3.1. Weight change of crab shell

Fig. 1 shows the weight change of sub- and supercritical water treated crab shell at (a) 400°C, (b) 350°C, and (c) 300°C. At 400ºC, the weight change of the crab shell decreased with treatment time and reached 37% at 5 min. At 350ºC, the weight change of the crab shell decreased drastically at 2 min, gradually decreasing to 54% at 10 min. At 300ºC, the weight change of the crab shell dropped at 5 min, after which it plateaued at around 70% until 40 min. Therefore, the final value of weight change after treatment with sub- and supercritical water decreased with increasing temperature. Additionally, the rate of weight change at 400ºC was greater than that at 350ºC and 300ºC.

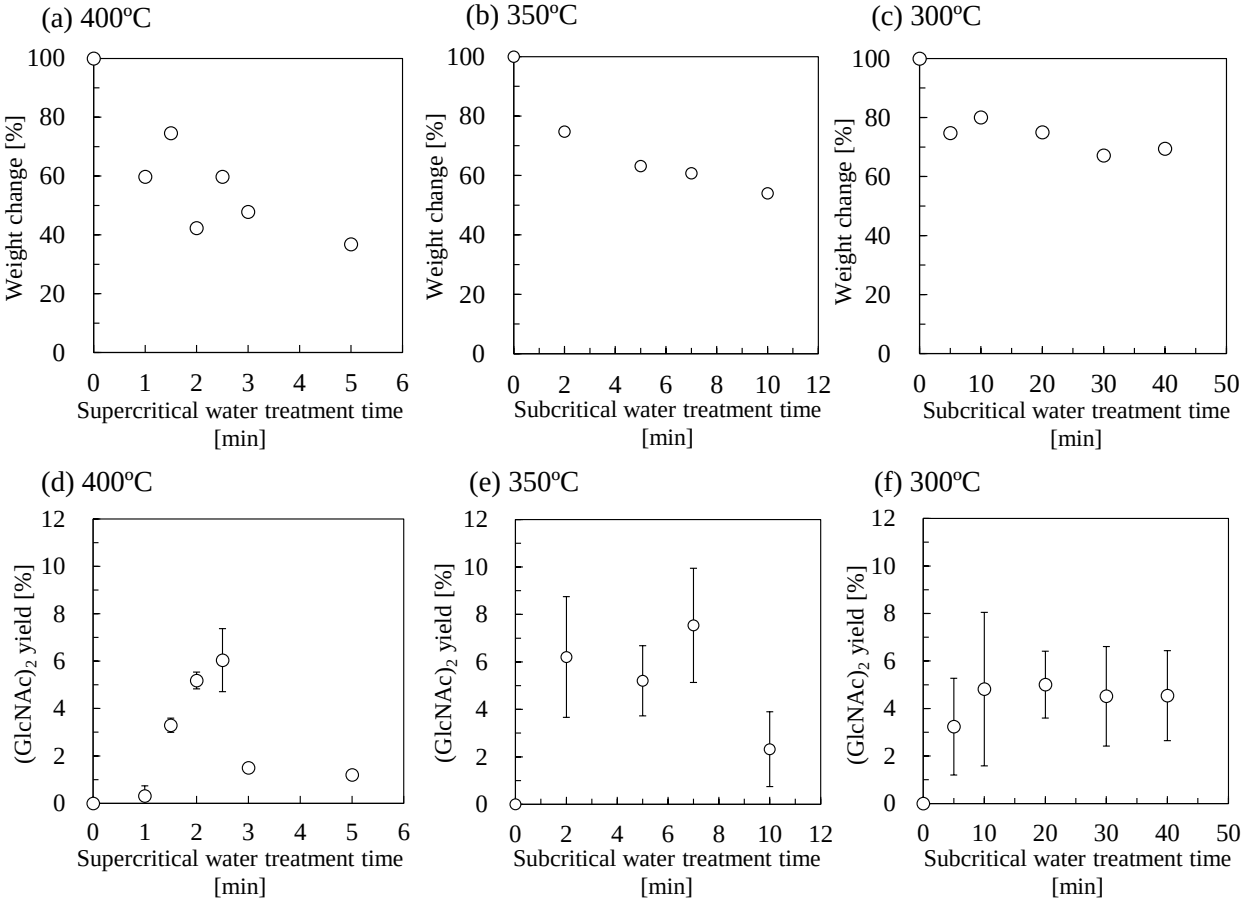


Fig. 1 Effect of sub- and supercritical water pretreatment time on weight change of crab shell and its enzymatic degradation (n = 3, bars indicate SEs).

3.2. Enzymatic degradation of crab shell

Fig. 1 (d), (e), and (f) show the effect of sub- and supercritical water treatment on the yield of $(GlcNAc)_2$ from crab shell after enzymatic degradation for 48 h. The crab shell contains approximately 30% α-chitin and therefore, the theoretical maximum $(GlcNAc)_2$ yield obtained by enzymatic degradation would be 30%. Zero min of treatment time means enzymatic degradation of untreated crab shell; however, it was not enzymatically degraded and the yield of $(GlcNAc)_2$ for 48 h was 0%. At 400ºC, the yield of $(GlcNAc)_2$ increased with increasing treatment time, reaching 6% at 2.5 min; it then decreased to approximately 1% at 5 min. At 350 ºC, the yield of $(GlcNAc)_2$ peaked at around 7 min, reaching 8%. At 300 ºC, the yield of $(GlcNAc)_2$ was almost constant after 10 min of treatment time. These results indicate that the optimum pretreatment temperature is 350ºC, for 7 min. The yields of GlcNAc from enzymatic degradation of untreated, and sub- and supercritical water treated crab shell for 48 h were less than 0.1%. Therefore, we focused our analysis on $(GlcNAc)_2$. We also analyzed the aqueous solution recovered after sub- and supercritical water treatment without subsequent enzymatic treatment. However, $(GlcNAc)_2$ and GlcNAc were not obtained under these conditions.

Fig. 2 shows the reaction time profile for enzymatic degradation of untreated and sub- and supercritical water treated crab shell at 400°C for 2.5 min and at 350°C for 7 min

those showed peaks of the yields of $(GlcNAc)_2$ in Fig. 1 (d) and (e). Untreated crab shell was not enzymatically degraded and the yield of $(GlcNAc)_2$ kept 0%. The yields of $(GlcNAc)_2$ after treatment with sub- and supercritical water at 400°C and 350°C increased with increasing the enzymatic reaction time, reaching 6 and 8%, respectively. Fig. 2 indicates that complete enzymatic degradation was achieved by 24 h.

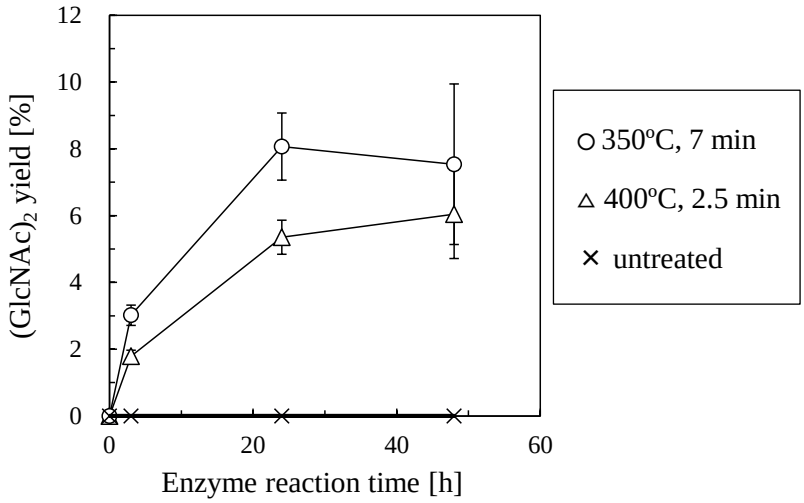


Fig. 2 Reaction time profile for enzymatic degradation of untreated, sub- and supercritical water treated crab shell (n = 3, bars indicate SEs).

3.3. X-ray diffraction (XRD)

Fig. 3 (a) shows the XRD patterns of untreated and supercritical water treated crab shell. For untreated crab shell, we observed a peak that was derived from α-chitin. The α-chitin peak became smaller with increasing treatment time and disappeared at 3.0 min. In contrast to this, the peak for $CaCO_3$ increased with increasing treatment time and only the

peaks of $CaCO_3$ could be observed at 5 min.

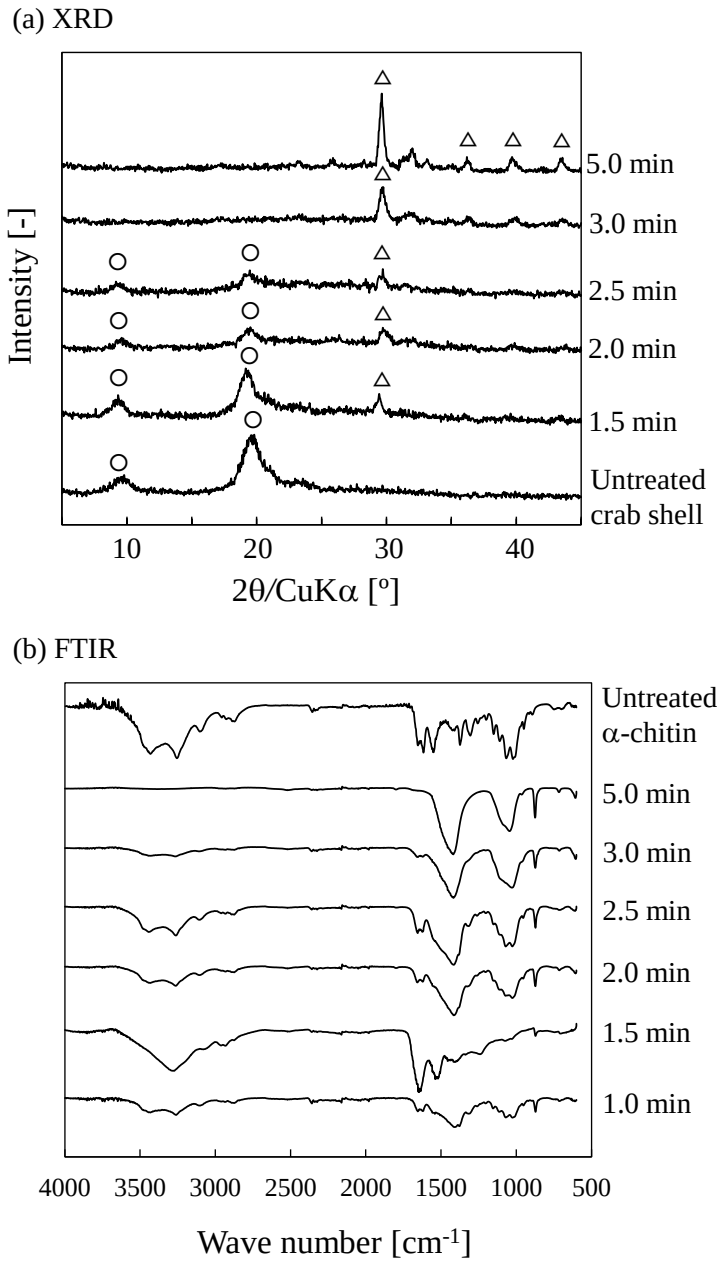


Fig. 3 Effects of supercritical water treatment at 400°C on the properties of crab shell: (a) XRD pattern (○: α-chitin, △: $CaCO_3$) and (b) FTIR spectra.

3.4. Fourier transform infrared (FTIR) spectroscopy

Fig. 3 (b) shows the FTIR spectra of supercritical water treated crab shell. The peaks around 3200–3500 cm^{-1} and 1500–1700 cm^{-1} are derived from α-chitin. The peaks around 1400 cm^{-1} and 870 cm^{-1} are derived from $CaCO_3$ in the crab shell. The peaks around 1000–1100 cm^{-1} are generally derived from ethers (C-O-C), namely β-1,4 bond of α-chitin. The FTIR spectrum at 1 min was a combination of α-chitin and $CaCO_3$. The peak of α-chitin increased at 1.5 min and decreased after 2 min. In contrast to this, the peak of $CaCO_3$ became greater after 2 min. At 5 min, the peak of α-chitin disappeared and only the peaks of $CaCO_3$ could be observed.

3.5. Weight change of pure α-chitin

Fig. 4 shows weight change of sub- and supercritical water treated α-chitin at (a) 400°C, (b) 350°C, and (c) 300°C. At 400ºC, the weight change of α-chitin decreased with treatment time and reached 5% at 2 min. The weight changes of α-chitin at 350ºC and 300ºC were 18% at 5 min and 33% at 30 min, respectively. The rate of weight change at 400ºC was faster than that at 350ºC and 300ºC. This trend is similar to that observed for the crab shell in Fig. 1 (a), (b), and (c). Interestingly, at 300ºC, the weight change of α-chitin decreased continuously, whereas that of the crab shell became constant after 5 min. A possible reason for this difference will be discussed later in the article.

3.6. Enzymatic degradation of pure α-chitin

Fig. 4 (d), (e), and (f) show the effect of temperature of sub- and supercritical water treatments on the yield of $(GlcNAc)_2$, after enzymatic degradation of α-chitin for 48 h. For α-chitin, the theoretical maximum $(GlcNAc)_2$ yield obtained from enzymatic degradation would be the same as the weight change value shown in Fig. 4 (a), (b), and (c). At 400ºC, the yield of $(GlcNAc)_2$ increased with increase in treatment time and reached 23% at 1 min, after

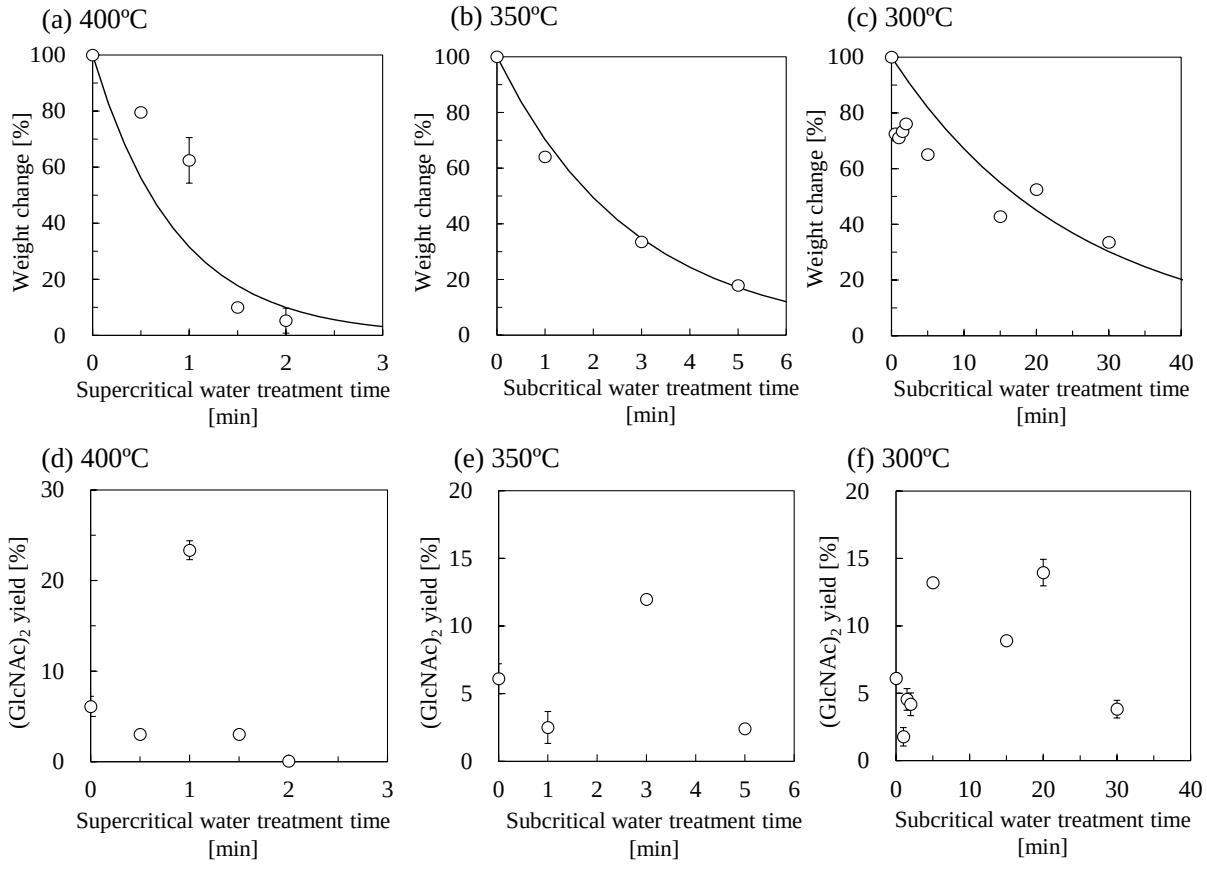


Fig. 4 Effect of sub- and supercritical water pretreatment time on weight change of α-chitin and its enzymatic degradation (n = 3, bars indicate SEs). The lines in (a), (b), and (c) are the first-order reaction fitting curves by eq. (3).

which it decreased to approximately 0% at 2 min. At 350ºC, the yield of $(GlcNAc)_2$ peaked at around 3 min, reaching 12%. At 300 ºC, the yield of $(GlcNAc)_2$ increased for 5 min and stayed constant at approximately 13% until 20 min, finally decreasing to 4% at 30 min. These results indicate that the optimum pretreatment time required for α-chitin at each treatment temperature is shorter than that required for crab shell, as shown in Fig. 1.

3.7. Mean molecular weight of pure α-chitin

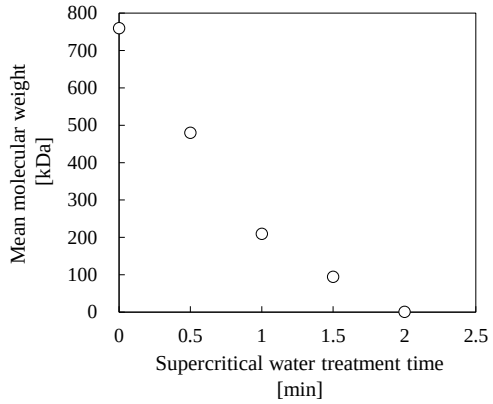
Fig. 5 (a) shows the effect of reaction time on the mean molecular weight of α-chitin in supercritical water at 400ºC. Untreated α-chitin flake (at 0 min) did not dissolve completely in the LiCl/DMAC solvent and we measured the mean molecular weight of only the soluble part of α-chitin. Therefore, the real mean molecular weight of untreated α-chitin would be greater than 760 kDa. On the other hand, α-chitin samples after supercritical water treatment dissolved completely in the LiCl/DMAC solvent. The mean molecular weight decreased with increasing treatment time and it was 0.9 kDa at 2 min.

3.8. Thermo gravimetric (TG) analysis of pure α-chitin

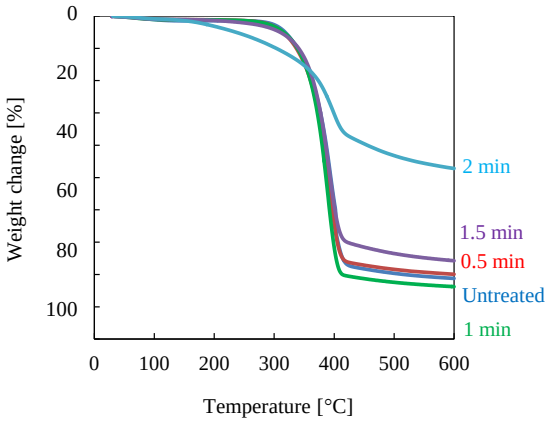
Fig. 5 (b) shows TG curves of untreated and supercritical water treated α-chitin at 400°C. The curves of untreated and supercritical water treated α-chitin at 0.5 to 1.5 min were almost the same, only changing at 2 min. These results indicate that the structure of treated

α-chitin until 1.5 min remained similar to untreated α-chitin. After 2 min, α-chitin changed to a carbonaceous material and the weight change as depicted by the TG curve decreased.

(a) Molecular weight



(b) Thermo gravimetric (TG) analysis



(c) Laser scanning microscopy

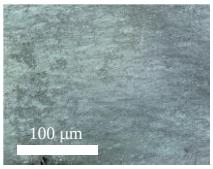
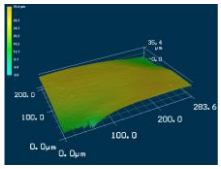
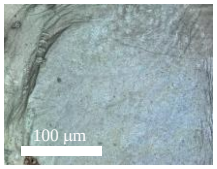
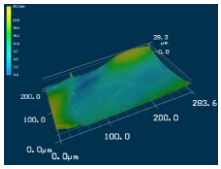
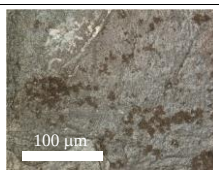
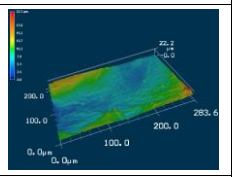
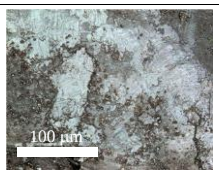
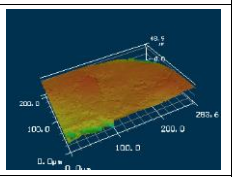
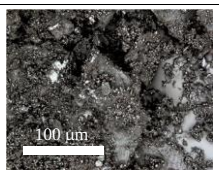
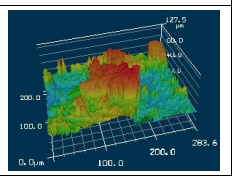
Treatment time (Average of surface roughness)	Surface image	Surface roughness
Untreated (0.2 μm)		
0.5 min (0.2μm)		
1 min (2 μm)		
1.5 min (3 μm)		
2 min (8 μm)		

Fig. 5 Effects of supercritical water treatment at 400°C on the properties of pure α-chitin: (a) mean molecular weight, (b) TG curves, and (c) surface roughness observed by laser scanning microscopy.

3.9. Laser scanning microscopy of pure α-chitin

Fig. 5 (c) shows the laser scanning microscopy image of supercritical water treated

α-chitin surface at 400°C. The surface roughness was low and under 3 μm until 1.5 min, increasing to 8 μm at 2 min. The trend of the surface roughness was similar to that of TG curve, as shown in Fig. 5 (b).

4. Discussion

4.1. Effects of sub- and supercritical water treatments on the properties of crab shell

As seen in Fig. 1 and 4, the weight change of crab shell was less than that of pure α-chitin at each temperature. Raw crab shell is composed of 30% α-chitin, 30% protein, and 40% $CaCO_3$. It has been reported that protein decomposes to amino acids through hydrolysis in subcritical water at around 200°C (Quitain et al., 2001). $CaCO_3$ is stable up to 400°C and 40 MPa, and is not soluble in supercritical water (Nakamura et al., 2007). Therefore, the change in the weight of crab shell at 300°C is mainly due to the decomposition of protein. At 400°C and 350°C, both α-chitin and protein were decomposed to water-soluble products. From XRD and IR analyses of the crab shell (Fig. 3), peaks derived from α-chitin could be observed until 2.5 min at 400°C. The peaks from $CaCO_3$ in XRD and IR analyses became greater after 3 min at 400°C, indicating that the main component of the solid residue was $CaCO_3$.

In enzymatic degradation of the crab shell, the peaks of $(GlcNAc)_2$ yield were observed at 2.5 min (at 400°C) and around 7 min (at 350°C). At 300°C, $(GlcNAc)_2$ yield did

not show a clear peak and remained at around 5%. These results indicate that the optimum sub- and supercritical water pretreatment time for crab shell for enzymatic degradation is after the removal of protein, but before the decomposition of α-chitin. At 300°C, the protein was hydrolyzed to amino acids and α-chitin was relatively stable. Therefore, the $(GlcNAc)_2$ yield showed a constant value. At 400°C and 350°C, we observed the decomposition of not only protein but also α-chitin after 2.5 and 7 min, respectively. This was followed by a decrease in $(GlcNAc)_2$ yield.

A comparison of weight change of crab shell and pure α-chitin indicated that the α-chitin contained in the crab shell was more stable than pure α-chitin. At 300°C, the weight change of crab shell after 5 min was about 70% (Fig. 1 (c)), which almost corresponds to the sum of $CaCO_3$ (40%) and α-chitin (30%). This indicated that α-chitin in the crab shell was almost stable at 300°C. However, the weight change of pure α-chitin decreased continuously with increasing treatment time at 300°C (Fig. 4 (c)). These results indicate that the physicochemical properties of α-chitin contained in the crab shell are different from those of pure α-chitin. The rate of changes observed in the properties of pure α-chitin as mentioned in section 4.2 would be slower for α-chitin contained in the crab shell. This is probably due to acid and base treatments used for α-chitin extraction. In this work, we found that the treatment time required for maximum $(GlcNAc)_2$ yield from crab shell was longer than that from pure α-chitin at each temperature. From the experimental results, the optimum treatment

times for crab shell at 400°C and 350°C are 2.5 and 7 min, respectively.

Although the optimum reaction time required for the treatment of crab shell was greater than that for pure α-chitin, the maximum $(GlcNAc)_2$ ratio based on the residual amount of chitin—as defined in our previous studies (Osada et al., 2012, 2013b)— was comparable for both crab shell and pure α-chitin. For example, the weight change of crab shell was 60% at 400°C and 2.5 min, assuming that 30% protein and 10% α-chitin decomposed. Therefore, 20% α-chitin remained and we obtained a $(GlcNAc)_2$ yield of 6%, making the $(GlcNAc)_2$ ratio based on the amount of residual chitin 30% (6/20 = 0.3). On the other hand, the weight change of pure α-chitin was 62% at 400°C and 1 min, indicating 38% pure α-chitin decomposed, and $(GlcNAc)_2$ yield of 23% was obtained. Therefore, the $(GlcNAc)_2$ ratio based on the amount of residual chitin was 37% (23/62 = 0.37). Similarly, $(GlcNAc)_2$ ratio based on the amount of chitin in the crab shell at 350°C and 7 min was 40% (8/20 = 0.4), and that in pure α-chitin at 350°C and 3 min was 35% (12/34 = 0.35). The $(GlcNAc)_2$ ratio based on the residual amount of chitin was approximately 30% to 40% for both crab shell or pure α-chitin, as discussed in Section 4.2. However, it should be noted that these calculations were based on assumptions of the residual amount of chitin in the crab shell.

4.2. Effects of sub- and supercritical water treatments on the properties of pure α-chitin

The decomposition rate of α-chitin in sub- and supercritical water was evaluated by assuming the first order reaction kinetics for the residual amount of α-chitin, *W(t)*, at time *t*.

$$dW(t)/dt = -k\,W(t) \quad (3)$$

where *k* is the overall reaction rate constant.

The results obtained in Fig. 4 (a), (b), and (c) were fitted with Eq. (3) to evaluate the rate constant, *k*. From the analysis of the solid residue of α-chitin by XRD and FT-IR (Osada et al., 2013b) and the laser scanning microscopy images in Fig. 5 (c), it could be concluded that α-chitin chemical structure was relatively stable, except supercritical water treatment at 400°C for more than 2 min. Therefore, we did not use these conditions to evaluate the rate constant, *k*.

The estimated rate constants are plotted against reciprocal temperature in Fig. 6. The kinetic parameters at temperature ranging from 300°C to 400°C such as the apparent activation energy, E_a, and the preexponential factor *A* were 108 kJ mol^{-1} and $10^{6.76}$, respectively. The decomposition rate of cellulose as reported in the literature (Peterson et al., 2008) is shown in Fig. 6; it was greater than that of α-chitin. Sakanishi et al. (1999) reported that *N*-acetyl group stabilized the α-chitin crystallite structure because of the additional hydrogen bond between the *N*-acetyl and OH group therefore, the hydrolysis of the β-1,4 bond of α-chitin was suppressed, as compared with cellulose. Some researchers have reported the decomposition rate of cellulose in sub- and supercritical water. However, similar reports

on α-chitin are limited in number. Aida et al. (2014) claimed that decomposition rate of α-chitin is greatly affected by particle size and crystallinity. Therefore, it should be noted that the data shown in Fig. 6 obtained from α-chitin of particle size approximately 3 mm × 3 mm × 0.5 mm and 90% crystallinity.

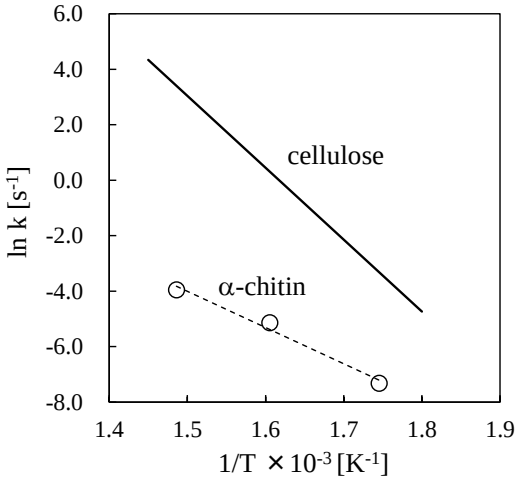


Fig. 6 Arrhenius plot overlay of first-order decomposition rate constants for the degradation of cellulose and α-chitin.

Interestingly, the decomposition rate of GlcNAc is greater than that of glucose (Osada et al. 2013a), which is opposite trend between α-chitin and cellulose. For GlcNAc, the dehydration proceeds between H-2 and OH-3 because the *N*-acetyl group is an electron-withdrawing group and the elimination of H-2 takes place easier, as compared with glucose. We also reported that the dehydration of $(GlcNAc)_2$ is promoted at the reducing end of $(GlcNAc)_2$ at lower temperatures of around 200°C, as compared with cellobiose (Osada et al., 2014). We have reported these findings in our previous studies that were focused on the dehydration of GlcNAc and $(GlcNAc)_2$ in subcritical water at around 200°C.

α-Chitin is almost stable at around 200°C as reported by Aida et al. (2014), whereas the dehydration of GlcNAc and $(GlcNAc)_2$ proceeds at the same temperature range. Since the dehydration rates of GlcNAc and $(GlcNAc)_2$ are greater than the hydrolysis rate of the β-1,4 bond of α-chitin, GlcNAc, $(GlcNAc)_2$, and chitin oligosaccharide cannot be obtained from α-chitin by sub- and supercritical water treatments on their own. Therefore, sub- and supercritical water pretreatments followed by enzymatic degradation of α-chitin or crab shell is a more promising method for the production of GlcNAc and $(GlcNAc)_2$.

Based on the experimental results of this work as well as previous studies, a schematic reaction of α-chitin in supercritical water at 400°C has been suggested in Fig. 7 From 0 to 0.5 min, the crystallite structures of α-chitin and the chemical structure of GlcNAc units are almost stable; these observations supported by XRD and IR analyses reported in our previous study (Osada et al., 2012). However, the hydrolysis of α-chitin continued and the molecular weight of α-chitin decreased gradually, as shown in Fig. 5 (a). At around 1 min, the distance between chitin chains increased, which is indicated by XRD analysis done in our previous study, and it is attributed an increased number of exposed amide group detected in the NIR spectra (Osada et al., 2013b). At around 1.5 min, water-soluble low-molecular weight chitin chains are formed because of the hydrolysis, and they are released from the chitin solid surface. At around 2 min, there was further hydrolysis of chitin chains and release of low-molecular weight chitin chains, which is attributed to the rough surface of chitin solid,

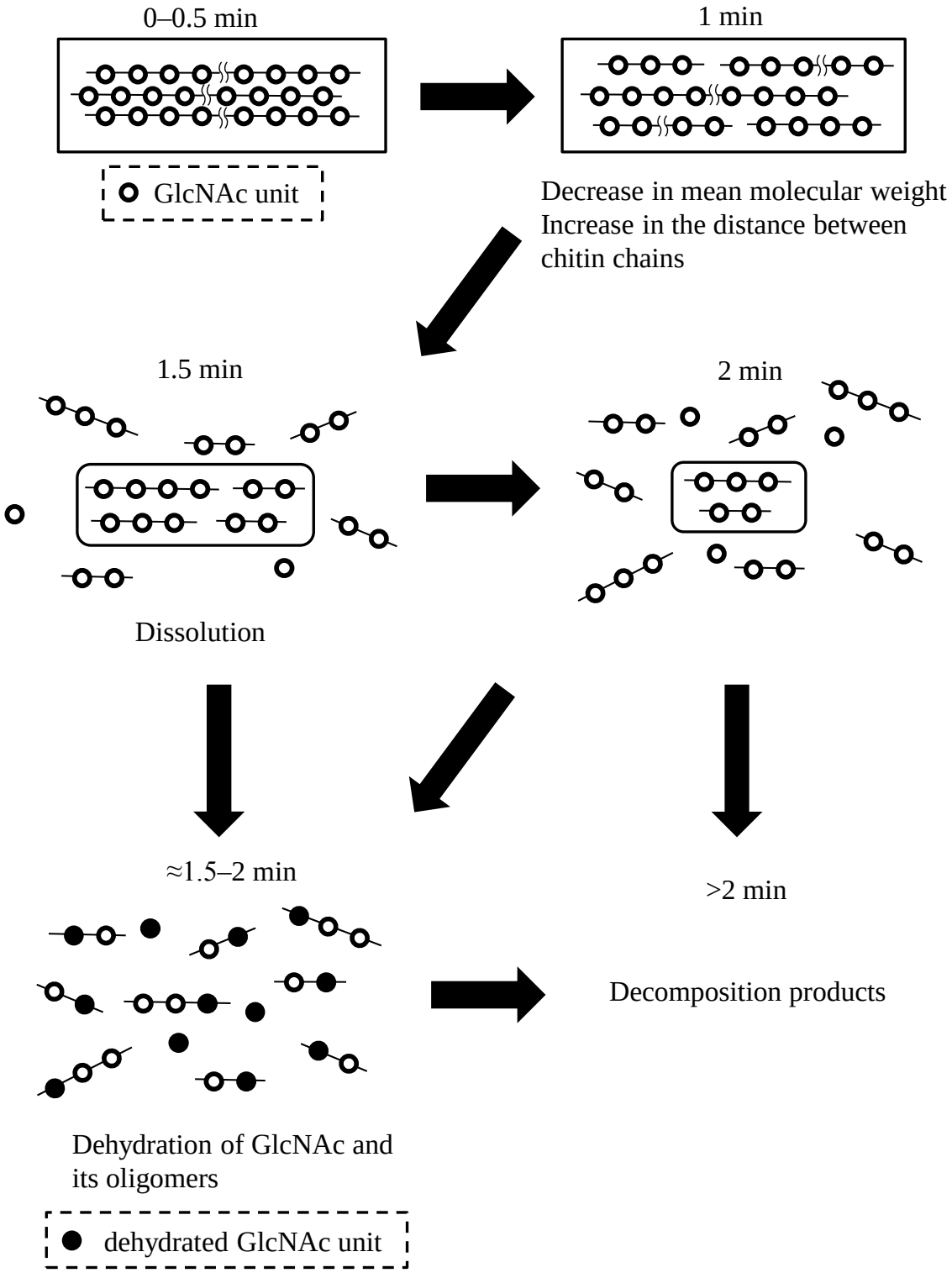


Fig. 7 Schematic of α-chitin reaction in supercritical water.

as shown in Fig. 5 (c). The low-molecular weight chitin chains are immediately dehydrated at the reducing end, reported in our previous study (Osada et al., 2014). We observed that the dehydration of $(GlcNAc)_2$ proceeded at temperatures above 180°C for 1 min, altering the chemical structure of GlcNAc unit. At a longer reaction time of 2 min, it has been reported that a condensation reaction between chitin decomposition products occurs, and water-insoluble solid products are formed. (Aida et al., 2014, Sakanishi et al., 1999).

The important changes in α-chitin properties that take place during pretreatment with sub- and supercritical water required for enzymatic degradation are (a) a decrease in mean molecular weight and (b) an increase in the distance between chitin chains. In this study, we used a batch type reactor, in which the treatment times included heat-up time. For a set temperature at 400°C, the actual temperature of the reactor at 1 min reached at about 380°C as we reported previously (Osada et al., 2012). Therefore, pure α-chitin was treated with both sub- and supercritical water within 1 min. In this work, 3.0g of water was loaded into 6 cm^3 of the reactor and an evaluation of water properties is possible. The ion product of water (K_w = $[H^+][OH^-]$) at 25°C is 1.0×10^{-14} mol^2 kg^{-2} and it increases with increasing temperature, reaches a maximum of 4.0×10^{-12} mol^2 kg^{-2} around 300°C above saturated vapor pressure of 9 MPa. Then the K_w decreases with increasing temperature and it became 1.9×10^{-13} mol^2 kg^{-2} at 400°C and 37 MPa. These K_w values mean that pure α-chitin was exposed from 10 to 100 times higher H^+ and OH^- concentrations condition during sub- and supercritical water

treatments than ambient condition. The combined effects of high H^+ and OH^- concentrations and high temperature are probably responsible for the hydrolysis of pure α-chitin and the weakened hydrogen bond between α-chitin chains observed in the absence of an added acid or base.

In this work, we found that the mean molecular weight decreased continuously through hydrolysis, as shown in Fig. 5 (a). We used a mixture of both endo- and exo-type enzymes. The decrease in the molecular weight leads to an increased number of end point of chitin chain and enhances a hydrolysable place by the exo-type enzyme. The increase in the distance between chitin chains indicates that the hydrogen bonds between hydroxyl groups of the chitin chains become weak. The weakened hydrogen bond groups increase the hydrophilicity of chitin, leading to easier access by enzymes. Therefore, the yields of $(GlcNAc)_2$ increased for up to 1 min at 400°C, as shown in Fig. 4 (d). After 1 min, the GlcNAc structure changed due to continuous dehydration and the enzymes could not hydrolyze dehydrated GlcNAc unit further. As mentioned in Section 4.1, the $(GlcNAc)_2$ ratio, based on residual amount of chitin, ranged from 30 to 40% for both crab shell and pure α-chitin. This is due to a kinetic balance between the hydrolysis of chitin chain and the dehydration of the GlcNAc structure.

5. Conclusions

This study demonstrates that sub- and supercritical water pretreatments significantly affect the physicochemical properties of crab shell and enhance the enzymatic degradation of crab shell. Sub- and supercritical water pretreatments removed the protein in the crab shell, whereas α-chitin and $CaCO_3$ remained stable for a certain reaction time and temperature. Prolonged treatment decomposed the α-chitin, and $CaCO_3$ was obtained as solid residue. These results indicate that, ideally, pretreatment of crab shell with sub- and supercritical water for enzymatic degradation should be done after the removal of protein, but before the decomposition of α-chitin. During sub- and supercritical water treatments, the mean molecular weight of pure α-chitin decreased and the distance between chitin chains increased due to weakening of hydrogen bonds. These changes of properties of α-chitin promoted enzymatic degradation. Subcritical (350°C, 7 min) and supercritical water (400°C, 2.5 min) treatments of crab shell, followed by enzymatic degradation, provided $(GlcNAc)_2$ yields of 8% and 6%, respectively. Without any pretreatment, the yield dropped to 0%. We found that crab shell requires a longer treatment time with sub- and supercritical water compared to pure α-chitin. In general, acid and base pretreatments of crab shell are required to remove $CaCO_3$ and protein before enzymatic degradation. This study shows that water treatment alone is adequate for the removal of protein and that changing α-chitin properties for producing GlcNAc and $(GlcNAc)_2$ from crab shell in an eco-friendly and sustainable manner is possible.

Although a small batch-type reactor was used in this work, development of a tubular flow reactor is needed to increase the treated crab shell amount.

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