



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 CaCO₃. 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.

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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, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 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, Matsui, & Isobe, 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 CaCO₃ 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 ($T_c = 374.3$ °C, $P_c = 22.1$ 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, Sato, Daimon, and Fujie (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

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claimed that glucosamine might decompose in sub- and supercritical water. Nakamura, Oozono, Nakai, and Yoshida (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 1–20 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 $(\text{GlcNAc})_2$ and GlcNAc from crab shell, because under these conditions, $(\text{GlcNAc})_2$ and GlcNAc decompose at the same time that the α -chitin is hydrolyzed (Aida et al., 2014; Sakanishi, Ikeyama, Sakaki, Shibata, & Miki, 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 $(\text{GlcNAc})_2$ 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 $\times$ 3 mm $\times$ 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:

Weight change (%)

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

2.4. X-ray diffraction (XRD)

Equatorial diffraction profiles of crab shell were obtained with Cu-K α 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, Miura, et al., 2013).

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:

Product yield (%)

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

The definition of product yield is different from our previous research (Osada et al., 2012; Osada, Miura, et al., 2013) 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 $(\text{GlcNAc})_2$ 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 figures 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 400 °C, the weight change of the crab shell decreased with treatment time and reached 37% at 5 min. At 350 °C, the weight change of the crab

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