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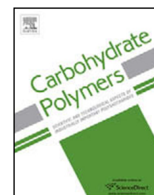

A simple procedure for preparing chitin oligomers through acetone precipitation after hydrolysis in concentrated hydrochloric acid

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ABSTRACT

Chitin oligomers are of interest because of their numerous biologically relevant properties. To prepare chitin oligomers containing 4–6 GlcNAc units [$(GlcNAc)_{4-6}$], α- and β-chitin were hydrolyzed with concentrated hydrochloric acid at 40 °C. The reactant was mixed with acetone to recover the acetone-insoluble material, and $(GlcNAc)_{4-6}$ was efficiently recovered after subsequent water extraction. Composition analysis using gel permeation chromatography and MALDI-TOF mass spectrometry indicated that $(GlcNAc)_{4-6}$ could be isolated from the acetone-insoluble material with recoveries of approximately 17% and 21% from the starting α-chitin and β-chitin, respectively. The acetone precipitation method is highly useful for recovering chitin oligomers from the acid hydrolysate of chitin. The changes in the molecular size and higher-order structure of chitin during the course of hydrolysis were also analyzed, and a model that explains the process of oligomer accumulation is proposed.

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1. Introduction

Chitin is linear polysaccharide that consists of β-(1,4)-linked *N*-acetyl-glucosamine (GlcNAc) and can be used as a raw material to produce chitin-derived products, such as chitosan, chitooligosaccharides, and glucosamine (GlcN). Chitin forms the carbohydrate backbone of crustacean and insect exoskeletons, and it is also found in the cell walls of microorganisms (Bowman & Free, 2006) and in the microfilarial sheaths of parasitic nematodes (Fuhrman & Piessens, 1985). Chitin also serves as a recognition element for tissue infiltration by the innate cells implicated in allergic and helminth immunity (Reese et al., 2007).

There is an increasing demand for chitin/chitosan oligomers in the health food and cosmetics industries. Chitin oligomers (*N*-acetyl-chitooligosaccharides) have various biological activities in plant, mammalian, and insect cells (Aam et al., 2010; Khoushab & Yamabhai, 2010). In cultured rice cells, *N*-acetyl-chitooligosaccharides with a degree of polymerization greater than five strongly activate various cellular reactions (Shibuya & Minami, 2001). In rat fibroblast cells, *N*-acetyl-chitohexaoses, $(GlcNAc)_6$, induce the continuous production of interleukin-8 (IL-8), a potent angiogenic factor and chemoattractant of polymorphonuclear cells (Mori et al., 1997). In silkworm, chitin oligomers form dimers to hexamers that trigger antibacterial protein expression as strongly as do lipopolysaccharides and peptidoglycans (Furukawa, Taniai, Yang, Shono, & Yamakawa, 1999). In addition, chitin oligomers specifically modified with sulfate, fatty acids, or some sugars play important roles as Nod factors that induce nodulation in legume roots in their symbiotic interactions with rhizobial bacteria (Truchet et al., 1991). These modified oligomers also play important roles as Myc factors that stimulate arbuscular mycorrhiza formation, root growth, and root branching in plants (Maillet et al., 2011).

Chitosan oligomers, which are homo- and heterooligomers of GlcN and GlcNAc, also have various biological activities and may function as antibacterial agents (Rhoades, Gibson, Formentin, Beer, & Rastall, 2006) and potential asthma drugs (Chung, Park, & Park, 2012). These oligomers may accelerate the elements of wound healing (You, Park, Ko, & Min, 2004), and they also have inhibitory effects on tumor growth and cancer metastasis (Shen, Chen, Chan, Jeng, & Wang, 2009). There are more reports on the biological effects of chitosan oligomers with varying degrees of polymerization than on the biological effects of chitin oligomers (Aam et al., 2010; Khoushab & Yamabhai, 2010).

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Chitin oligomers are produced using various procedures. Rupley hydrolyzed chitin with concentrated hydrochloric acid (HCl) under various conditions, and each chitin oligomer up to $(GlcNAc)_5$ could be separated from the hydrolysate to prepare substrates for reacting with lysozyme (Rupley, 1964). Cabrera et al. described a procedure involving the reacetylation of chitosan oligomers with acetic anhydride (Cabrera, Messiaen, Cambier, & Cutsem, 2006). Sakai et al. suggested a more efficient procedure, the sugar elongation method (Sakai, Nanjo, & Usui, 1990). They prepared $(GlcNAc)_6$ and $(GlcNAc)_7$ through a transglycosylation reaction using lysozyme from $(GlcNAc)_2$ as an initial substrate. In contrast, Samain et al. proposed a biotechnological procedure in which the cultivation of *Escherichia coli* harboring different combinations of heterologous *nod* genes led to the production of $(GlcNAc)_5$, tetra-*N*-acetyl-chitopentaose [GlcN-$(GlcNAc)_4$], and sulfated and *O*-acetylated derivatives through oligosaccharide biosynthesis from the UDP-GlcNAc pool (Samain, Drouillard, Heyraud, Driguez, & Geremia, 1997; Samain, Chazalet, & Geremia, 1999).

Because chitin is the second most abundant natural biopolymer, it is readily available as a starting material to prepare chitin oligomers. To date, however, the yield of chitin oligomers has been low, and their availability has been rather limited. A simpler and more efficient method for preparing chitin oligomers would improve this situation and help in clarifying the molecular machinery involved in the interactions of biological systems with chitin oligomers.

In this study, we developed a simple procedure for recovering chitin oligomers by using acetone precipitation rather than the neutralization method after the HCl hydrolysis of chitin (α-chitin) from crab shell. Chitin oligomers containing 4–6 GlcNAc units were recovered in good yield from the acetone precipitate by extraction with water. We also hydrolyzed chitin (β-chitin) from squid pen under similar conditions and compared the results with those obtained using α-chitin.

2. Materials and methods

2.1. Materials

Chitin (α-chitin) from crab shell and chitin (β-chitin) from squid pen were purchased from Tokyo Chemical Industry (Tokyo, Japan) and from Katakura Chikkarin Co. Ltd. (Tokyo, Japan), respectively. Unless otherwise noted, all reagents used were of the highest quality available from Wako Pure Chemicals (Osaka, Japan) or Sigma–Aldrich (St. Louis, MO, USA).

2.2. Characterization of chitin

The chitin samples were powdered in a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA) to a particle size of 250 μm. The weight-averaged molecular weight (M_w), the number-averaged molecular weight (M_n) and the molecular weight distribution of the chitins were determined using gel permeation chromatography (GPC). The GPC system consisted of a JASCO PU-980 pump (JASCO, Tokyo, Japan), a JASCO CO-965 column oven (JASCO), and Shodex KD-804 and KD-803 columns (8.0 mm × 300 mm, Showa Denko, Tokyo, Japan) at 50 °C. The mobile phase solvent used for the analysis was 5% LiCl/*N*,*N*-dimethylacetamide (DMAc) (w/v) at a flow rate of 0.5 mL/min. The effluent was monitored using a JASCO RI-930 refractive index detector (JASCO). The M_w and M_n were estimated by referring to the retention time of the TSK standard polyethylene oxide (Tosoh, Tokyo, Japan).

2.3. Determination of the degree of chitin deacetylation

The degree of deacetylation (DD) of chitin was determined by elemental analysis. The elemental analysis of chitin was performed at the Analytical Center of the Tokyo College of Pharmacy.

2.4. X-ray diffraction analysis of chitin

X-ray diffraction patterns of chitin were recorded using a MiniFlex X-ray diffractometer (Rigaku, Tokyo, Japan). The experiments used a Cu Kα target at 30 kV and 15 mA. The relative intensity was recorded over the scattering range (2θ) of 5.0°–35° with scan steps of 0.02° at a scan speed of 4.0°/min.

2.5. Chitin hydrolysis by concentrated HCl

To hydrolyze chitin using concentrated HCl, we followed Rupley and Vårum's method with a slight modification (Rupley, 1964; Vårum, Ottøy, & Smidsrød, 2001). Chitin (200 mg) was hydrolyzed in concentrated HCl (8.0 mL) under reflux at 40 °C for 5, 15, 30, 45, or 60 min.

2.6. Recovery of residues by the neutralization method after chitin hydrolysis

Following the hydrolysis of chitin by concentrated HCl, 6 M NaOH (16 mL) was added to the reaction mixture to stop the hydrolysis, and molarity of diluted NaOH was added to adjust the solution pH to 4–5. The neutralized suspension was centrifuged at 15,000 × *g* for 10 min, and the precipitate was washed with water. To completely remove the salt, washing was repeated several times until no precipitate appeared upon the addition of $AgNO_3$. Next, to prevent the chitin from forming a hydrogen bond and recrystallizing, the precipitate was washed twice with acetone in a similar manner. To obtain a constant weight, the precipitate was then suspended with diethyl ether, centrifuged, air-dried overnight and further dried in a vacuum desiccator over P_2O_5.

2.7. Recovery of residues by acetone precipitation after chitin hydrolysis

Following the hydrolysis of chitin by concentrated HCl, the reaction mixture was poured into 200 mL of acetone, and the mixture was stirred for 1 day at 4 °C. After the mixture was centrifuged at 10,000 × *g* for 10 min at 4 °C, the precipitate was washed several times with acetone to remove HCl until the pH of the supernatant reached 4–5. To obtain a constant weight, the precipitate was suspended with cold diethyl ether, centrifuged, air-dried overnight, and further dried in a vacuum desiccator over P_2O_5.

To extract the chitin oligomers from the acetone precipitate, the dried acetone precipitate was soaked in 5 mL of water and stirred overnight at 20 °C. After centrifugation at 15,000 × *g* for 10 min at 20 °C, the precipitate was soaked in 2 mL of water and then stirred overnight at 20 °C. Both supernatants were combined and used as water-soluble oligomers. The water-insoluble material was washed with acetone, washed with diethyl ether, air-dried overnight, and then further dried in a vacuum desiccator over P_2O_5 until a constant weight was obtained.

2.8. Analysis of water-extracted chitin oligomers

GPC was performed on an HPLC system, which consisted of a Waters 510 pump (Waters, Milford, MA, USA), a CTO-6A column oven (Shimadzu, Kyoto, Japan), a SPD-6A UV-detector (Shimadzu) and a Shodex GS-220 HQ column (7.5 mm × 300 mm, Showa Denko, Tokyo, Japan). The separation was performed at 50 °C using H_2O as

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