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Preparation and characterization of α-chitin from cicada sloughs

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ABSTRACT

In this study, a new source of insect chitin was proposed. Insect chitin was extracted from cicada sloughs by 1 M HCl and 1 M NaOH treatment for demineralization and deproteinization, respectively. The brown color of this chitin from cicada sloughs was removed using 6% sodium hypochlorite as an oxidizing agent. It was found that the insect chitin extracted from the cicada sloughs has a higher percent recovery than the chitin from rice-field crab shells. The chemical structure and physicochemical properties of α-chitin from cicada sloughs were characterized using elemental analysis (EA), attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), proton nuclear magnetic resonance spectroscopy (^{1}H NMR), solid-state ^{13}C cross-polarization magic-angle-spinning nuclear magnetic resonance (CP/MAS) NMR spectroscopy, X-ray diffractometry (XRD), and thermogravimetry (TG). The degree of acetylation (DA) was determined by EA, ^{1}H NMR, and ^{13}C CP/MAS NMR techniques. The DA values of chitin from cicada sloughs were in the range of 97% to 102% depending on each technique. Furthermore, it was found that the DA increased with an increasing thermal property and crystallinity.

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1. Introduction

A cicada is an insect of the order homoptera, suborder auchenorrhyncha, in the superfamily cicadoidea. It is characterized by its large eyes which are wide apart on the head and its usually transparent well-veined wings. There are about 2500 species of cicada around the globe and many remain unclassified. Cicadas live in temperate to tropical climates where they are among the most widely recognized of all insects, mainly due to their large size and remarkable acoustic talents [1]. Cicada sloughs are predominantly chitin, a polysaccharide that is a structural component found in the cell walls of fungi and in the exoskeletons of insects and other arthropods. Chitin consists of β-(1,4)-linked-2-acetamido-2-deoxy-D-glucopyranose units (GlcNAc). There are three crystalline forms of chitin: α-, β-, and γ-chitin [2]. Naturally, the α-crystalline form is the most abundant and the molecules are aligned in an antiparallel fashion. Moreover, the α-crystalline form is the most stable form of the three crystalline variations. It is found in the shells of crustaceans, the skeletons of insects, and the cell walls of fungi [3]. The β-crystalline form is rarely found in nature and the molecules are packed in a parallel arrangement, leading to weaker intermolecular forces than those in α-chitin. The β-crystalline form is found in squid pens, in the extracellular spines of the euryhaline diatom, and in pogonophore tubes [4]. The γ-crystalline form has a mixture of antiparallel and parallel chains and is found in the cocoons of insects [5]. However, the traditional commercial sources of chitin are crab, shrimp, and krill shells that constitute waste from the processing of marine food. Recently, chitin has been obtained from unconventional sources such as insects and fungal mycelia [6–8]. Normally, in the preparation of chitin from crustacean sources, the conditions must be treated with strong concentrated acid and alkali in order to remove calcium carbonate and protein. However, insect cuticles rarely contain inorganic materials, therefore, a very low concentration of acid and alkali is required [6]. Nevertheless, there have been few reports on the preparation and characterization of chitin from insects. Previous studies have mainly focused on crab and shrimp chitin. However, studies on the structure of insect chitin have recently been carried out on beetle larva cuticle and silkworm (*Bombyx mori*) pupa exuvia [6]. Other sources of insect chitin are those isolated from the honeybee (*Apis mellifera*) [7], bumblebee (*Bombus terrestris*) [9], scorpion, spider, brachiopod, ant, and cockroach [10]. In this study, a new source of insect chitin from cicada sloughs is isolated and characterized. Moreover, the physicochemical properties of chitin were determined by elemental analysis, ATR-FTIR, ^{1}H NMR, solid-state ^{13}C cross-polarization magic-angle-spinning nuclear magnetic resonance (CP/MAS) NMR spectroscopy, thermogravimetry, X-ray diffractometry, and scanning electron microscopy. The relationship between the DA, thermal property and crystalline index (CrI) is discussed. These results are then compared with those of the chitin of rice-field crab shells (*Somanniathelphusa dugasti*). Four species of rice-field crabs (*Somanniathelphusa*), *S. germaini*, *S. sinensis*, *S. juliae* (Bott), and *S. dugasti* (Rathbun), are found throughout Thailand [11]. The over-infestation of *S. dugasti* in northern Thailand damages rice stalks, reducing rice production so people in the north of the country eat it as food.

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2. Experimental

2.1. Materials

The rice-field crab shells and cicada sloughs were obtained from Thailand. The rice-field crab shells and the cicada sloughs were washed with distilled water and dried in an oven at a temperature of 50 °C for 24 h. Then they were mechanically ground in a mixer and passed through a gauze.

2.2. Preparation of chitin from cicada sloughs

The chitin from the crab shells was prepared from rice-field crab shells according to the Hackmann method [12,13] while the insect chitin was isolated from the cicada sloughs as described by Zhang et al. [6]. Briefly, 30 g of cicada sloughs were treated with 1 N HCl at 100 °C for 20 min then rinsed with distilled water until neutrality was reached. After that, the solid product was treated with 1 N NaOH at 80 °C for 36 h, then filtered and refluxed with 0.4% Na_2CO_3 for 12 h. Next, the chitin product was washed with distilled water until the pH became neutral. The pigment of this product was removed using 6% sodium hypochlorite as the oxidizing agent. Finally, the chitin of the cicada sloughs was washed with distilled water again and dried in vacuum. The final chitin powder obtained was 11 g.

2.3. Chitin characterization

2.3.1. Infrared spectroscopy and elemental analysis

All attenuated total reflectance-Fourier transform infrared spectrophotometer (ATR-FTIR) spectra were collected with a Nicolet 6700 spectrometer (Thermo Company, USA) using single-bounce ATR-FTIR spectroscopy (Smart Orbit accessory) with a diamond internal reflection element (IRE) at ambient temperature (25 °C). Spectra were collected using standard spectral collection techniques and the rapid-scan software in OMNIC 7.0. In all cases spectra were collected using 32 scans with a resolution of 4 cm^{-1}. Elemental analysis was performed using a PE 2400 CHNS/O analyzer (Perkin Elmer, USA). The percentage of protein remaining in chitin samples was determined using Eq. (1) [14–16].

$$\text{Protein}\,(\%) = [N(\%) - 6.9] \times 6.25 \quad (1)$$

where $N(\%)$ is the total nitrogen content measured by elemental analysis, 6.9 is the theoretical content of nitrogen in chitin, and 6.25 is the theoretical average content of nitrogen in proteins.

The average degree of acetylation (DA) of the chitin samples was determined from the data of elemental analysis, as shown in Eq. (2) [17].

$$\text{DA} = [(\text{C}/\text{N} - 5.14)/1.72] \times 100 \quad (2)$$

where C/N is the ratio (w/w) of carbon to nitrogen.

2.3.2. ^1H NMR spectroscopy and ^{13}C cross-polarization magic-angle-spinning (CP/MAS) NMR spectroscopy

The ^1H NMR spectra were measured on an Avance AV 500 MHz spectrometer (Bruker, Switzerland). The measurements were performed at 300 K, using a pulse accumulation of 64 scans and an LB parameter of 0.30 Hz. D_2O/DCl was used as the solvent for dissolved chitin samples at 50 °C. The DA was calculated according to the following Eq. (3) [18,19].

$$\text{DA} = (\text{NHAc}/3)/[(\text{NHAc}/3) + \text{H2 GlcN}] \times 100 \quad (3)$$

where NHAc and H2 GlcN are the integrals area of the three protons of the *N*-acetyl group and the integrals area of H-2 of the glucosamine unit, respectively.

^{13}C cross-polarization magic-angle-spinning (CP/MAS) NMR spectra (Bruker, Switzerland) were recorded on an Avance 300 MHz Digital NMR spectrometer (Bruker Biospin; DPX-300, Switzerland). All samples were characterized at 20 ± 1 °C and recorded at a frequency of 75 MHz. The spectral parameters used were as follows: 1600 number of scans, relaxation delay of 4 s, spin rate of 5 kHz, and spectral size 2 K with 4 K time size. The DA of chitin samples was calculated from the relative intensities of the resonance of the ring carbon (*I*C1, *I*C2, *I*C3, *I*C4, *I*C5, *I*C6) and methyl carbon ($I\text{CH}_3$) which were obtained from ^{13}C NMR spectra using the following Eq. (4) [20–22].

$$\text{DA} = [I\text{CH}_3/(I\text{C1} + I\text{C2} + I\text{C3} + I\text{C4} + I\text{C5} + I\text{C6})/6] \times 100 \quad (4)$$

2.3.3. X-ray diffractometry

The X-ray diffraction (XRD) measurement was applied to determine the crystallinity of the chitin samples and their patterns were recorded using a JEOL JDX-3530 theta-2theta X-ray diffractometer (Aremco Products, Inc., USA) with CuKα radiation (λ = 1.5406). The 2θ angle was scanned between 4° and 40° and the counting time was 2.0 s at each angle step. The voltage was 40 kV and the intensity 30 mA. The crystalline index (CrI; %) was determined in two ways as shown in the following Eqs. (5) and (6) [23].

$$\text{CrI}_{020} = [(I_{020} - I_{\text{am}})/I_{020}] \times 100 \quad (5)$$

where I_{020} is the maximum intensity at $2\theta \cong 9°$ and I_{am} is the intensity of amorphous diffraction at $2\theta \cong 16°$.

For comparison, another crystalline index was expressed as CrI_{110}, following the equation above and using I_{110} as shown in Eq. (6).

$$\text{CrI}_{110} = [(I_{110} - I_{\text{am}})/I_{110}] \times 100 \quad (6)$$

where I_{110} is the maximum intensity at $2\theta \cong 20°$.

2.3.4. Thermogravimetry analysis

The thermogravimetric analysis (TGA) was recorded by TGA/SDTA 851 (METTLER TOLEDO) with a heating rate of 10 °C/min from 25 °C to 1000 °C under nitrogen atmosphere.

2.4. Ash and moisture contents

The ash content of the sample was determined by storing the sample in a muffle furnace at 600 °C for 4 h or until the weight was constant. The ash content was calculated from the percentage of ash residue compared to the initial weight of the moisture-free sample. The moisture content of the sample was determined by drying the sample at 105 °C for 24 h or until the weight was constant. It was then calculated by the percentage of weight loss compared to the initial weight of the sample. The lipid content was estimated after a soxhlet extraction with chloroform/methanol (2/1, v/v) and subsequent gravimetric analysis of the sample [24]. The surface morphology was visualized by a scanning electron microscope (SEM) (Hitachi, S-3400 N, Japan) with a combined energy dispersive X-ray (EDX) analyzer (Horiba; EMAX, Japan) which determined the metals contained in the chitin samples.

3. Results and discussion

3.1. Preparation of the chitin from cicada sloughs

The chitin from cicada sloughs was purified by demineralization with hydrochloric acid, deproteinization with sodium hydroxide, and decolorization with sodium hypochlorite, respectively. The recovery of purified chitin from the cicada sloughs was 36.6% whereas that of the rice-field crab shells was 15.2% (Table 1). The cicada sloughs consisted of 8.7% moisture, 11.7% ash, 2.7% lipid and lipoprotein, 39.8%

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