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Isolation and characterization of chitin from bumblebee (*Bombus terrestris*)

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Abstract

Insect chitin possessing shell-like structure was prepared from the bumblebee corpses by a consequent treatment with 1 M HCl and 1 M NaOH. The bumblebee chitin was compared with crustacean (shrimp) chitin by using elemental analysis, Fourier-transform infrared (FT-IR) and solid-state ^{13}C cross-polarization magic angle spinning nuclear magnetic resonance (CP/MAS)-NMR spectroscopy and confocal microscopy. Both chitins (bumblebee and shrimp) exhibited identical spectra, while the bumblebee chitin had a 5% lower degree of acetylation and was characterized by a fine membrane texture.

Keywords: Chitin; Bumblebee; Shrimp; Spectroscopy; Microscopy

1. Introduction

Chitin the second most abundant after cellulose biopolymer found in nature after, is produced by many living organisms, and is present usually in a complex with other polysaccharides and proteins. Chitin was found as a major component in arthropods (insects, crustaceans, arachnids and myriapods), nematodes, algae and fungi [1–4].

Chitin is a linear polysaccharide composed of (1 → 4)-linked 2-acetamido-2-deoxy-β-D-glucopyranosyl units and occurs naturally in three polymorphic forms with different orientations of the microfibrils, known as α-, β-, and γ-chitin [1,5]. The α-chitin has antiparallel chains and is a common and the most stable polymorphic form of chitin in nature, which is prevalent in crustaceans and in insect chitinous cuticles [6–8]. The β-form of chitin is rare, it occurs in pens of mollusks and is characterized by a loose-packing parallel chains fashion with weak intermolecular interactions and higher solubility and swelling than α-form; β-chitin was prepared from the pens of the squid *Ommastrephes bartrami* [9,10], and *Loligo* species and cuttlefish (*Sepia officinalis*) [6,11–13]. The γ-form is characterized by a mixture of antiparallel and parallel chains and was found in the cocoons of insects [14].

Chitin is not soluble in usual solvents, whereas chitosan – its most common derivative obtained by a partial deacetylation of chitin – becomes soluble in aqueous acidic solutions and behaves as a cationic electrolyte when the degree of acetylation (DA) is lower than 0.5 [15].

Chitin and chitosan are biomolecules of a great potential, both possessing versatile biological activities, demonstrating excellent biocompatibility and complete biodegradability [16]. Therefore, they have found extensive applications in pharmacy, medicine, agriculture, food and textile industries, cosmetics, and wastewater treatment [4,17–21].

The insect chitin was isolated from beetle larva cuticle and silkworm (*Bombyx mori*) pupa exuvia and physicochemically characterized as α-form [22]. Another insect chitin was isolated from honeybee (*Apis mellifera*) by Nemtsev et al. [23].

In the present paper we describe isolation and characterization of chitin from bumblebee (*Bombus terrestris*) corpses and comparison of its physicochemical properties with the crustacean chitin.

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2. Materials and methods

2.1. Materials

Frozen bumblebee corpses, which arose from the biotechnological production of bumblebees used for vegetable and fruit pollination, were purchased from Vega Konti, s.r.o. (Pata, Slovakia). Commercially available chitin prepared from fresh shrimp shells (*Pandalus borealus*) was purchased from Primex (Siglufjordur, Iceland).

2.2. Preparation of chitin from bumblebees

Bumblebee corpses were washed several times with water and dried at 50 °C overnight in a dry heat incubator. Afterwards, they were mechanically grinded in a mixer and passed through four layers of gauze. Demineralization step was carried out by a treatment with 1 M HCl at 100 °C for 20 min, which also resulted in removal of the catechols. The demineralization step was followed by rinsing with distilled water until neutrality was reached. Deproteinization was performed using alkaline treatment with 1 M NaOH solution at 85 °C. This treatment was repeated several times during 24 h. The chitin product was filtered through four layers of gauze with the aid of vacuum pump and washed with distilled water until the pH became neutral. Pigment traces responsible for the brown color of this product were removed using a mild oxidizing treatment (H_2O_2/33% HCl 9:1, v:v) [15]. Finally, lightly brown chitin was washed with distilled water and dried at 50 °C in a dry heat sterilizator. The structure of chitin and its purity were evaluated by using ^{13}C solid-state NMR and FT-IR spectroscopy and electron microscopy.

2.3. Elemental analysis

Elemental analyses were performed using an EA 1108 CHNO analyzer (Fisons Instruments, San Carlos, CA) at the Institute of Chemistry, Slovak Academy of Sciences, Slovakia.

2.4. FT-IR measurements

Measurements were carried out on a Nicolet Magna 750 FT-IR spectrophotometer equipped with triglycine sulfate (TGS) based detector employing a KBr pellet method. Absorbance values were determined between 4000 and 400 cm^{-1} with a resolution of 4 cm^{-1}. The analyses were performed using Omnic 3.1 statistical software package (Thermo Electron Scientific Instruments Corp., Madison, WI). KBr discs were prepared in a conventional way from the dry mixtures of about 1 mg of the sample and 100 mg of KBr. The DA of both chitin samples was determined by comparing the absorbance of the measured peak (proportional to the DA) to that of the reference peak (independent of the DA). The DA was calculated from the absorbance (A) ratios by using the method of Baxter et al. [24] according to the following equation:

$$\mathrm{DA}\,(\%) = \frac{A_{1655}}{A_{3450}} \times 115 \qquad (1)$$

2.5. Solid-state ^{13}C CP/MAS-NMR spectroscopy

^{13}C cross-polarization magic-angle-spinning (CP/MAS)-NMR spectra were recorded at 125.8 MHz on a Bruker Avance 500 spectrometer with a spinning rate of 10 kHz and using a contact time of 2 ms. A total of 6144 scans were acquired for each run, and measurements were performed at room temperature.

The DA of the samples was determined by dividing the intensity of the resonance of the methyl group carbon by the average intensity of the resonances of the glycosyl ring carbon atoms. The DA was calculated using the following relationship [25]:

$$\mathrm{DA}\,(\%) = \frac{100I[\mathrm{CH_3}]}{(I[\mathrm{C1}] + I[\mathrm{C2}] + I[\mathrm{C3}] + I[\mathrm{C4}] + I[\mathrm{C5}] + I[\mathrm{C6}])/6}$$

(I is the intensity of the particular resonance peak) (2)

2.6. Confocal microscopy

The microscopic observation was performed using a confocal microscope IMAGER.Z1 (Zeiss Axioimager.Z1, Carl Zeiss MicroImaging GmbH, Göttingen, Germany). A small amount of the chitin preparation was added to a drop of Fluoromount-G (Southern Biotechnology Associates Inc., Birmingham, AL) on a slide and mounted with a cover slip, pressed gently with tissue paper to remove excess of mounting medium, left to settle for 5–10 min at room temperature, and sealed with nail varnish.

3. Results

3.1. Elemental analysis

The experimentally obtained values of nitrogen content (%) of both chitin samples were lower than the theoretical value (6.89%) calculated for a completely acetylated chitin. Since the nitrogen content is also indicative of the residual protein still present in chitin, a low experimental value implied the minimum amount of protein left.

The difference between the nitrogen content of shrimp and bumblebee chitins was in agreement with estimated DA values for both chitins obtained by FT-IR and ^{13}C CP/MAS-NMR spectroscopy (Table 1).

3.2. FT-IR spectroscopic analysis

The FT-IR spectra (Fig. 1) of shrimp α-chitin and bumblebee chitin were very similar. The absorption peaks at 1540 cm^{-1}, assigned to the stretching vibration of protein [26], were absent.

Table 1
Results of the elemental analysis (%) of the chitin samples

Samples	N	C	H
Bumblebee chitin	5.92	43.92	6.43
Shrimp chitin	4.85	44.72	6.47

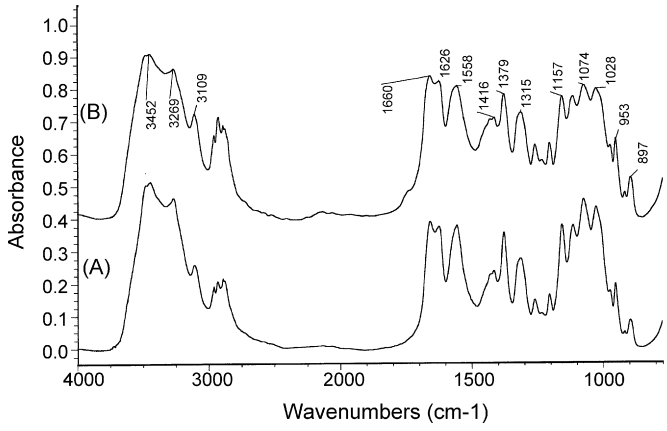

Fig. 1. FT-IR spectra of bumblebee chitin (A) and of shrimp α-chitin (B) (in KBr pellets).

This confirms that the prepared bumblebee chitin was protein free. In its spectrum, the three amide bands ascribed to the CONH group vibrational modes appeared at 1659 cm^{-1} (amide I), 1558 cm^{-1} (amide II) and 1315 cm^{-1} (amide III). Four intensive bands ascribed to the C–O–C and C–O stretching modes appeared at 1157, 1117, 1076 and 1028 cm^{-1}. The vibrational absorption band at 1379 cm^{-1} is assigned to the rocking of the methyl group [26]. Table 2 shows the assignment of the relevant bands of bumblebee chitin based on the assignment made for shrimp α-chitin. The DA values of bumblebee and shrimp α-chitin calculated using the formula (2) were 87.3% and 99.0%, respectively.

Table 2
Assignments of the relevant bands of FT-IR spectra of bumblebee chitin and of shrimp α-chitin

Assignments	Wave number (cm^{-1})	
	Bumblebee chitin	Shrimp α-chitin
ν(O–H)	3444	3452
$\nu(COCH_3)$	2933	2933
ν(C–H)	2891	2891
ν(C–O)	1659	1660
ν(C═O of *N*-acetyl group)	1626	1626
δ(N–H of *N*-acetyl group)	1558	1558

ν: stretching; δ: bending.

3.3. ^{13}C CP/MAS-NMR spectroscopic analysis

Solid-state ^{13}C NMR spectroscopy is known to be very sensitive to changes in the local structure. ^{13}C CP/MAS-NMR spectra of the chitin samples are shown in Fig. 2. NMR analysis of the bumblebee chitin sample gave similar peak patterns to that of the commercial shrimp α-chitin. Each spectrum consisted of eight well-defined resonances of C-1–C-6 carbons of the *N*-acetylglucosamine monomeric unit, which were observed between 50 and 110 ppm, indicating high structural homogeneity. The CH_3 group of the acetyl group of chitin samples gave rise to a signal at 22.5 ppm, while its carbonyl group produced a peak at 173 ppm. The ^{13}C signals for C-5 (75.49 ppm) and C-3 (73.08 ppm) in the ^{13}C CP/MAS-NMR spectrum of bumblebee chitin were clearly separated into two signals (Fig. 2), similarly to the shrimp α-chitin. The recorded resonances were

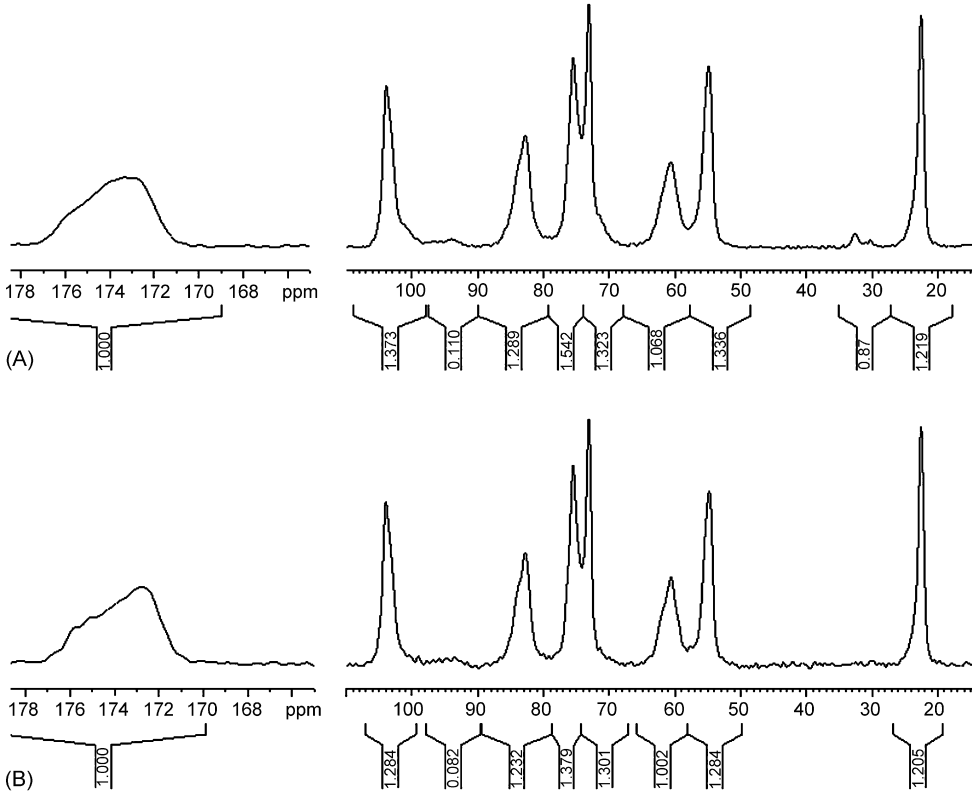

Fig. 2. ^{13}C CP/MAS-NMR spectra of bumblebee chitin (A) and of shrimp α-chitin (B).

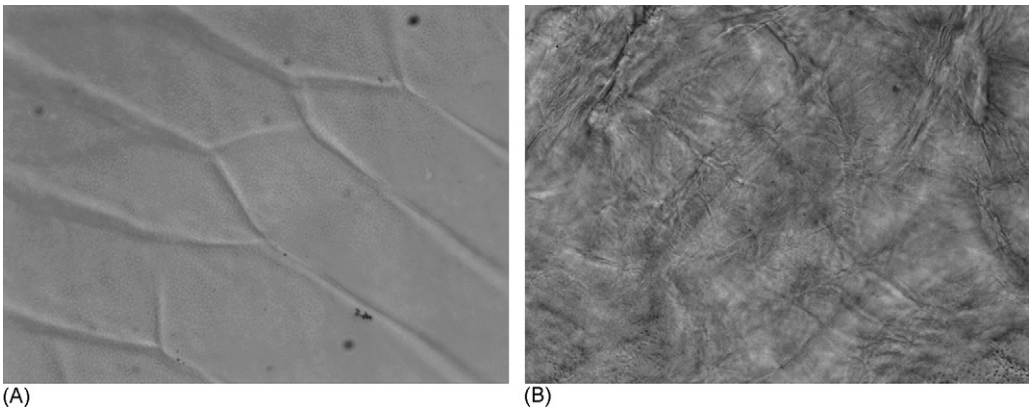

Fig. 3. Confocal microscopy photographs of bumblebee chitin (A) and of shrimp α-chitin (B).

Table 3
^{13}C CP/MAS-NMR spectral data of the bumblebee and crustacean chitin samples

^{13}C signal assignment	Chitin samples	
	Bumblebee chitin	Shrimp α-chitin
C═O	173.34	172.76
C-1	103.91	103.91
C-4	82.75	82.78
C-5	75.49	75.49
C-3	73.08	73.09
C-6	60.59	60.60
C-2	54.87	54.78
CH_3	22.52	22.55

very close to the expected values (Table 3). The additional signal for the bumblebee chitin was probably due to the traces of catechol compounds (32.57 ppm) still present in the preparation. The DA values of bumblebee chitin and the untreated shrimp α-chitin calculated applying the formula (1) were 90.9% and 95.6%, respectively.

3.4. Confocal microscopy

The used isolation procedure of bumblebee chitin allowed to obtain a product of high chemical purity, which possessed a shell-like structure. The microscopic observation showed that these shells were forming a fine regular hexagonal structure similar to that of the original bumblebee cuticule (Fig. 3A). In contrast to this fine insect chitin, the crustacean (shrimp) chitin was rougher and the microscopic picture reflected its amorphous character (Fig. 3B).

4. Discussion

Chitin is a major component of the insect cuticle and serves as a light but mechanically strong scaffold material. Chitin is always associated with cuticle proteins that determine the mechanical properties of the cuticle. The antiparallel arrangement of chitin molecules in the α-form may contribute significantly to the physicochemical properties of the cuticle [7]. In the family Apiidae, chitin creates the cuticle, in which internal organs are placed protected by this barrier against the environment. The interior of a cuticle is formed by hypodermis, which contains different proteins that together with chitin provide high firmness, flexibility and elasticity.

The traditional commercial sources of chitin are crab, shrimp, and krill shells that constitute wastes from the processing of marine food products. In the recent years, more importance is gained by chitin obtained from unconventional sources such as insect and fungal mycelia [22,23,27].

Insect corpses do not contain inorganic material, therefore, only mild demineralization treatment is required to recover insect chitin, which has lower degree of acetylation also the rate of hydrolysis of the insect chitin by chitinases is higher than that of the crustacean chitin [22].

The DA is the most important characteristic of chitin and its value depends on the source material and the preparation methods used. Solid-state ^{13}C CP/MAS-NMR spectroscopy appears to be suitable for the evaluation of the degree of acetylation [28] and is known to be very sensitive to changes in the local structure. It does not require the solubilization of the polymer, but presumes high degree of purification of the samples studied. NMR spectra of the chitin isolates from fungal mycelia of *Aspergillus niger* [27] significantly differed in the distribution of the C-1–C-6 carbon signals and methyl group resonance in comparison with the bumblebee chitin. This fact may suggest that fungal chitin contains higher amount of residual proteins.

At investigating the shrimp and bumblebee chitins, higher values of DA were obtained by NMR spectroscopy than by FT-IR analysis, which is in agreement with previously published assessment of these methods [29].

Chitin yield from the dried bumblebee corpses is lower than that from the crustaceans. On the other hand, better physico-mechanical properties (fine texture) of the bumblebee chitin and chitosan predestinate them for use as a homogenous dispersion in a protective colloid (e.g. prepared from pectin or alginate) for biomedical purposes.

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