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Three-dimensional chitin rings from body segments of a pet diplopod species: Characterization and protein interaction studies

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ABSTRACT

Physicochemical characterization of new chitin isolates can provide valuable insights into designing of biomimetic materials. Chitin isolates with a definite three-dimensional (3D) structure can exhibit characteristics that distinguish them from other chitin specimens that are in form of powder or flakes without a definite and uniform shape. Herein, 3D chitin rings were produced from body segments of a diplopod (*Archispirostreptus gigas*) inhabiting tropical regions. This organism is cultured easily and can reach 38 cm in length, which makes it a suitable source for isolation of chitin. The chitin rings were characterized via TGA, FT-IR, SEM and XRD analyses. Enzymatic digestion test with chitinase demonstrated that chitin isolates had high purity (digestion rate: 97.4%). The source organism had high chitin content; 21.02 ± 2.23% on dry weight. Interactions of the chitin rings with bovine serum albumin (BSA) protein were studied under different conditions (pH: 4.0–8.0, chitin amount: 6–14 mg, contact time: 30–360 min, protein concentration: 0.2–1 mg/mL). The highest BSA adsorption was observed at pH 5.0 at 20 °C. The adsorption equilibrium data exhibited a better fit to Langmuir adsorption and the pseudo-first order kinetic models. The findings presented here can be useful for further studies aiming to develop biocompatible and nontoxic biomaterials.

1. Introduction

Chitin is a structural biopolymer found in the biosphere abundantly. Its presence in the exoskeleton structures of many invertebrates [1,2], cell walls of fungi [3–5] and diatoms [6,7] has already been acknowledged by many literature reports. Still, chitin itself and its derivatives are attracting much interest, and a recent interesting study has reported the presence of chitin synthase gene in certain vertebrates [8]. So far, crustacean exoskeletons from waste of food processing industry have been exploited as a main source for commercial chitin production [9–11]. Some recent studies have demonstrated that chitin isolates from other sources such as fungi [5] and insect cuticles [12–14] exhibit different characteristics that deserve a special attention. Especially, some recent studies reported by Ehrlich et al. have provided new insights into the nature and function of chitin found in the organisms of phylum Porifera [15–18]. The authors isolated three dimensional chitin structures from sponges, and they had excellent results regarding applications of those chitin isolates [19–22].

Chitinous scaffolds with definite 3D structure exhibit unique properties such as biocompatibility, high hydration capacity and porous surfaces with interconnected channels that are suitable for diffusion of molecules or cell growth. These properties make 3D chitin isolates a promising material for biomedicine and bioengineering studies [19]. However, literature review revealed that 3D chitin production is limited to the sponges, and chitin from organisms of the class Diplopoda (Arthropoda) has not been studied in detail.

Diplopods have segmented bodies and their body segments can be a suitable raw material for 3D chitin production. The species *Archispirostreptus gigas* is one of the largest diplopods, reaching up to 38.5 cm in length and 6.7 cm in circumference [23]. This species lives in regions from Mozambique to Kenya, and it has also a cosmopolitan distribution in east African coast limited to altitudes of below 1000 m [24,25]. This organism has a relatively large and segmented body, it can be cultured easily and it is also known as a pet. These were the determinative qualities in selection of *A. gigas* as a model organism in this study.

Serum albumin proteins are abundantly found in the blood plasma of many organisms and they play vital roles in the delivery of many bioactive molecules and substances throughout the body [26]. These

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proteins interact reversibly with biological molecules and carry them to the cells. Therefore, their interaction with biocompatible surfaces is of fundamental importance [27]. Interactions of modified chitin or chitosan with blood proteins have been elucidated in earlier reports [28–31]. However, blood protein interaction studies of native chitin are very limited and therefore a detailed study is needed for further research in chitin-based biomimetic materials designing.

In present study the interaction of the chitin rings from *A. gigas* with BSA protein was investigated. 3D chitinous matrices from *A. gigas* were isolated and characterized using analytical tools such as thermogravimetric analysis (TGA), Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM) and X-ray diffraction (XRD) analyses. Hydrolytic digestion of the chitin isolates with chitinase enzyme was also conducted.

2. Materials and methods

2.1. Materials

Fully-grown individuals of *A. gigas* were purchased from a local pet shop and were shipped to the laboratory in 70% ethanol solution. The body segments of the organisms were separated manually, washed with distilled water and oven-dried at 50 °C for 1 week. Dried body segments were kept in sealed containers at 4 °C in a refrigerator. Chitinase from *Streptomyces griseus* (EC. 3.2.1. 14), commercial α-chitin from shrimp shell (pcode: 1001416772), HCl (conc., 37%), ethanol and the salts (Na_2CO_3, NaH_2PO_4 and Na_2HPO_4) were purchased from Sigma-Aldrich. $K_3[Fe(CN)_6]$ was obtained from Merck.

2.2. Extraction of the chitin from diplopod body segments

Body segments of *A. gigas* were subjected to 5% NaClO solution at 50 °C for 10 min and then washed thoroughly with distilled water. This treatment was highly efficient in removal of pigments from the structure, and as a result of this treatment the color of the segments turned into white from brownish-yellow. Bleached samples were treated with 1 M HCl solution for 1 h at room temperature. The bubbles that were formed during acid treatment of the samples can be ascribed to the high mineral content of the samples. Following the acidic treatment, the samples were rinsed with distilled water. In the last step, the samples were treated by 1 M NaOH solution at 50 °C for 12 h. Finally, the samples were thoroughly rinsed with distilled water. The wet rings were oven-dried at 50 °C for one week. Chitin content of the body segments was calculated on dry basis.

2.3. Characterization of 3D chitin rings

Characterization of 3D chitin obtained from the diplopod was conducted by using TGA, FT-IR, SEM, XRD and Brunauere-Emmette-Teller (BET) analyses and chitinase digestive test.

Infrared spectrum of 3D chitin was recorded on a Perkin-Elmer FT-IR spectrometer (frequency range: 4000–625 cm^{-1}). Thermogravimetric analysis of 3D chitin was conducted on an EXSTAR S11 7300 (heating rate: 10 °C min^{-1} from room temperature to 750 °C, pan type: platinum, amount of the sample: 10 mg) under nitrogen atmosphere. 3D chitin was coated with gold by using "Sputter Coater" (Cressingto Auto 108) and was analyzed with QUANTA - FEG 250. X-ray diffractogram of 3D chitin was recorded on Rigaku model powder X-ray diffractometer with Cu *K* alpha radiation (40 kV, 30 mA, and at 2θ, scan angle: 5°–45°). The crystalline index value (CrI) of 3D chitin was calculated by using the formula below;

$$CrI_{110} = [(I_{110} - I_{am})/I_{110}] \times 100 \quad (1)$$

$$CrI_{020} = [(I_{020} - I_{am})/I_{020}] \times 100 \quad (2)$$

where I_{110} denotes maximum intensity at $2\theta \cong 20°$, I_{020} is maximum intensity at $2\theta \cong 9°$ and I_{am} is intensity of amorphous diffraction at $2\theta \cong 16°$.

Both 3D and commercial chitin samples were grinded by using commercial blender. The obtained dust samples were sieved by using 40 μm mesh size net to ensure homogenous size distribution for crystallinity analysis. Crystallinity measurements were taken the same conditions using the same XRD analysis tool and equipment as mentioned above.

3D chitin obtained from the diplopod was treated with chitinase from *S. griseus*. To quantify the enzymatic hydrolytic products spectrophotometrically, the potassium ferricyanide assay was employed [32], in which the amount of $K_4[Fe(CN)_6]$ present in the solution is proportional to the amount of hydrolytic products with reducing sugar.

Chitin powder from *A. gigas* or commercial chitin (100 mg) was incubated in 2 mL of conc. HCl acid solution and kept at 4 °C for 24 h to arrest hydrolysis of chitin. Following the addition of 2.5 mL of water and 2.5 mL of ethanol, the mixture was vigorously stirred on a magnetic stirrer for 10 min at room temperature. Then, the mixture was transferred into dialysis tubing (Viskase Sales Crop, Seamless Cellulose Tubing, Size: 16/32, 100 ft, Lot: 208001) and dialyzed against water until reaching neutral pH. Then, the solution was dialyzed against phosphate buffer solution (pH 7.0, 10 mM). Finally, 2.0 mL of colloidal solution from the dialysis bags was transferred into a test tube containing 1.0 mL of chitinase solution in phosphate buffer (0.5 mg/mL, buffered at pH 7.0, 10 mM) and incubated at room temperature for 72 h.

Following the incubation, the solution was kept in boiling water for 5 min to terminate the enzymatic reaction and centrifuged at 8000*g* for 15 min. One milliliter of supernatant was mixed with 1 mL of the reagent (it was prepared by dissolving 0.025 g of $K_3[Fe(CN)_6]$ in 0.5 L of 0.5 M Na_2CO_3 solution) and heated in boiling water for 20 min. The mixture was then centrifuged at 8000*g* for 15 min. The supernatant was assayed on a UV–vis spectrophotometer (Shimadzu, Tokyo, Japan, Model 1601) at 420 nm and the amount of the reducing end groups from hydrolysis of chitin was calculated colourimetrically from the decrease in the absorbance of anion $[Fe(CN)_6]^{3-}$.

Surface characteristics of chitin rings were examined on a Brunauere-Emmette-Teller (BET) analyzer (Micromeritics TriStar II 3020 nitrogen adsorption/desorption apparatus).

2.4. Protein interaction studies of 3D chitin rings

Interactions of the 3D chitin rings with BSA protein were tested under the following conditions: pH (4.0–8.0), chitin amount (6–14 mg), contact time (30–360 min) and protein concentration (0.2–1.0 mg/mL). All the experiments were conducted at 20 °C and pH of the protein solutions was adjusted by using two different buffer systems i.e., pH 4.0 and 5.0; 0.01 M acetate buffer and pH 6.0, 7.0 and 8.0; 0.01 M phosphate buffer. Briefly, chitin samples were put in 10 mL of protein solution and then agitated on a platform shaker (New Brunswick Scientific Excella E5). Protein concentration in the final solution was determined on the basis of absorbance measurements at 280 nm on a UV visible spectrophotometer (Beckman Coulter DU-730). The amount of protein adsorbed by 3D chitin was calculated from the difference in protein concentration using the following equation [33];

$$q_e = (C_i - C_f)V/m_c \quad (3)$$

where C_i and C_f are the initial and final protein concentration (mg/mL), q_e is amount of protein adsorbed by chitin rings (mg g^{-1}), V is the volume of the protein solution (mL), and m_c is the mass of chitin (g).

3. Results and discussion

3.1. Chitin content

The studied diplopod species (*A. gigas*), its body segments and obtained chitin rings are shown in Fig. 1. The chitin content of body