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Structural differences between chitin and chitosan extracted from three different marine sources

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ABSTRACT

Three marine sources of chitin from Tunisia were investigated. Structural differences between α-chitin from shrimp (*Penaeus kerathurus*) waste, crab (*Carcinus mediterraneus*) shells, and β-chitin from cuttlefish (*Sepia officinalis*) bones were studied by the ^{13}C NMR, FTIR, and XRD diffractograms. The ^{13}C NMR analysis showed a splitting of the C3 and C5 carbon signals for α-chitin, while that of β-chitin was merged into a single resonance. The bands contour of deconvoluted and curve-fit FTIR spectra showed a more detailed structure of α-chitin in the region of O—H, N—H and CO stretching regions. IR and ^{13}C NMR were used to determine the chitin degree of acetylation (DA). XRD analysis indicated that α-chitins were more crystalline polymorph than β-chitin. Shrimp chitin was obtained with a good yield (20% on raw material dry weight) and no residual protein and salts. Chitosans, with a DA lower than 20% and relatively low molecular masses were prepared from the wet chitins in the same experimental conditions. They were perfectly soluble in acidic medium. Nevertheless, chitin and chitosan characteristics were depending upon the chitin source.

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1. Introduction

Chitin is the second most abundant polysaccharide in biomass after cellulose. Chitin production was about 25,000 tons in 2006 [1]. This polymer is a linear chain consisting of poly β(1–4) N-acetyl-D-glucosamine. Chitin is usually isolated from the exoskeletons of crustaceans and more particularly from shrimps and crabs where α-chitin isomorph is produced [2]. α-Chitin has a tightly compact structure due to its crystalline structure in which antiparallel chain favor strong hydrogen bonding [3]. Squid is another important source of chitin in which it exists in the β-isomorph which was found to show higher solubility, higher reactivity and higher affinity toward solvents and swelling than α-chitin. These characteristics are due to weaker intermolecular hydrogen bonding ascribable to the parallel arrangement of the main chains [4]. γ-Chitin, a third chitin allomorph, has also been described [5,6], but from a detailed analysis, it seems that it is just a variant of the α-family [7].

Several techniques to extract chitin from different sources have been reported. The most common method is referred to the chemical procedure which involves various major steps. Firstly, shells are ground and minerals, mainly calcium carbonate, are removed (demineralization) using dilute acidic medium (usually HCl) at ambient temperature. Secondly, the proteins are extracted (deproteinization) from the residual material by treatment with aqueous solutions of NaOH or KOH.

These traditional chemical methods create a disposal problem due to the large amounts of toxic waste which would pollute the environment. In addition, this process becomes expensive due to enforced environmental controls and disposal measures [8]. To overcome the disadvantage of chemical treatments, some efforts have been directed toward its substitution by more eco-friendly processes such as bacterial fermentation [9] or treatment by proteolytic enzymes which have been applied for the deproteinization of crustacean wastes [9–12].

Because solid state chitin has a compact structure, it remains insoluble in most solvents. Therefore, usually chemical deacetylation is performed to produce the most common derivative named chitosan [13]. In acidic conditions, chitosan becomes positively charged due to $-NH_2$ protonation and soluble in aqueous medium. Under these conditions, this polymer has numerous physiological and biological properties with great potential in a wide range of industries such as cosmetology (lotions, hair additives, body creams) [14,15], food (coating, preservative, antioxidant,

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antimicrobial) [16], biotechnology (chelator, emulsifier, flocculent) [17], pharmacology and medicine (fibers, drugs, membranes, artificial organs) [18] and agriculture (soil modifier, films, fungicide) [19].

Chitosan can be characterized in terms of its quality, intrinsic properties (purity, molar mass, viscosity, and acetylation degree) and physical forms [20]. It was reported that both acetylation degree and molar mass are important chemical characteristics, which could influence the performance of chitosan in many of its applications [21].

The aim of this work was to extract chitin from different Tunisian marine sources using chemical demineralization and enzymatic deproteinization (with *Bacillus mojavensis* A21 proteases), and then to compare their physicochemical characteristics using XRD, FTIR and ^{13}C NMR spectroscopy. Following, the chitins obtained were deacetylated to chitosans. Acetylation degree and molar mass of each chitosan were examined. To the best of our knowledge, this present work is the first systematic trial to investigate the extraction of chitin and chitosan from different indigenous sources in Tunisia.

2. Materials and methods

2.1. Preparation of raw material

Shrimp (*Penaeus kerathurus*) waste, crab (*Carcinus mediterraneus*) shells and cuttlefish (*Sepia officinalis*) bones were obtained in fresh conditions from a fishery products and processing plant located at Sfax, Tunisia. They were washed thoroughly with tap water, dessicated at room temperature and milled (sieved from 2 mm to 5 mm). After drying, they were kept at room temperature until used.

2.2. Chemical analysis of the raw materials

The moisture and ash contents were determined at 105 °C and 550 °C, respectively, according to the AOAC [22] standard methods 930.15 and 942.05. Total nitrogen content of each raw material was determined by using the Kjeldahl method. Separately, for each raw sample, pure chitin is prepared to determine its nitrogen contribution allowing to estimate the crude protein content by multiplying nitrogen content attributed to protein by the factor of 6.25 [23]. Lipids were determined gravimetrically by soxhlet extraction using hexane.

2.3. Chemical demineralization

Demineralization was carried out at room temperature using 0.55 M hydrochloric acid baths. Each bath was performed with 100 ml of acid solution and 10 g of raw material. The number of baths and their duration (between 15 and 60 min) were dependent upon the source [24]. Demineralization step was followed by pH evolution toward neutrality due to acid consumption. The end of the repeated series of baths was indicated by stability of medium acidity. Demineralized materials were filtered through four layers of gauze using a vacuum pump, washed to neutrality with deionized water and dried for 1 h at 60 °C. Demineralization degree (DDM) was expressed as a percentage and computed by the following equation [25]:

$$\%\mathrm{DDM} = \frac{[(A_\mathrm{O} \times O) - (A_\mathrm{R} \times R)]}{A_\mathrm{O} \times O} \times 100 \quad (1)$$

where A_O and A_R are ash contents (%) before and after demineralization, respectively, while O and R represent the mass (g) of initial and demineralized residue respectively on dry weight basis.

2.4. Crude enzyme preparation

B. mojavensis A21 was isolated from marine water in Sfax by Haddar et al. [26]. The growth medium used for protease production was composed of (g/L): hulled grain of wheat, 30.0; yeast extract, 1.0; $CaCl_2$, 2.0; $MgSO_4$, 1.0; K_2HPO_4, 0.3; KH_2PO_4, 1.0; and NaCl, 2.0. The pH medium was adjusted to 9.0. Media were autoclaved at 121 °C for 20 min. Cultivations were conducted in 250 ml Erlenmeyer flasks with a working volume of 25 ml. Incubations were carried out in an orbital shaking incubator at 30 °C and 200 rpm for 24 h. The cultures were centrifuged at 10,000 rpm for 15 min and the cell-free supernatants were recovered and concentrated by the addition of solid ammonium sulfate to 80% saturation. Protease activity was measured by the method described by Kembhavi et al. [27] using casein as a substrate.

2.5. Deproteinization of shell waste by proteases

B. mojavensis A21 crude enzyme preparation was selected for its deproteinization efficiency [10]. Deproteinization tests were carried out in a thermostated stirred Pyrex reactor (300 ml). The demineralized shells homogenate (15 g) were mixed with 45 ml distilled water. The pH and temperature of the mixture were adjusted to pH 9.0 (with NaOH 4%, w/v) and 50 °C. The shell waste proteins were digested with crude enzyme using different Enzyme/Substrate ratios (E/S) during 3 h. The reaction was stopped by heating at 90 °C for 20 min to inactivate enzymes. The solid phase was washed, pressed manually through four layers of gauze and then dried at 60 °C during 12 h. Deproteinization degree (DDP) was expressed as percentage and computed by the following equation [25]:

$$\%\mathrm{DDP} = \frac{[(P_\mathrm{O} \times O) - (P_\mathrm{R} \times R)]}{P_\mathrm{O} \times O} \times 100 \quad (2)$$

where P_O and P_R are the protein concentrations (%) before and after hydrolysis, respectively, while O and R represent the mass (g) of original sample and hydrolyzed residue respectively on dry weight basis.

2.6. Deacetylation of chitin

Purified chitin was treated with 12.5 M NaOH in 1:10 (w/v) ratio at 140 °C for 4 h until it was deacetylated to a chitosan perfectly soluble in might acidic conditions. After filtration, the residue was washed with deionised water, and the crude chitosan was recovered by drying in a dry heat incubator at 50 °C during 12 h.

2.7. Physicochemical characterization of chitins and chitosans

2.7.1. Fourier transform infrared spectroscopy (FTIR)

Infrared spectra were obtained using a Perkin Elmer type FTIR 1000 spectrometer at room temperature and KBr pellets. The sample pellets were prepared at a pressure of 5 tons for 2 min. Pellets were scanned at room temperature (25 °C) in the 400–4000 cm^{-1} spectral range [28]. The incertitude of this measurement was 2 cm^{-1}. In the case of overlapping peaks, deconvolution was performed to calculate the contribution of the individual peaks using Dmfit program [29] with Gaussian and/or Lorentzian shapes to estimate the area related to the specific vibration of each selected peak. Spectra were corrected for the baseline and the absorbance was normalized between 0 and 1.

The acetylation degree (DA) of chitins and chitosans was calculated according to the method proposed by Moore and Roberts [30], as follows:

$$\mathrm{DA}\ \ (\%) = \frac{A_{1650}/A_{3450}}{1.33} \times 100 \quad (3)$$

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