International Journal of Biological Macromolecules 87 (2016) 163–171

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Chitin and chitosan from the Norway lobster by-products: Antimicrobial and anti-proliferative activities

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ARTICLE INFO

Article history:
Received 7 December 2015
Received in revised form 24 January 2016
Accepted 23 February 2016
Available online 24 February 2016

Keywords:
Norway lobster
By-products
Chitin and chitosan
Antimicrobial activity
Anti-proliferative property

ABSTRACT

Chitin was recovered through enzymatic deproteinization of the Norway lobster (*Nephrops norvegicus*) processing by-products. The obtained chitin was characterized and converted into chitosan by *N*-deacetylation, the acid-soluble form of chitin. Chitosan samples were then characterized by Fourier transform infrared spectroscopy (FTIR) and 13 Cross polarization magic angle spinning nuclear magnetic resonance (CP/MAS)-NMR spectroscopy. The antimicrobial activity and anti-proliferative capacity of chitosan were evaluated.

Antimicrobial activity assays indicated that prepared chitosan exhibited marked inhibitory activity against the bacterial and fungal strains tested. Further, cytotoxic effects of chitosan samples on human colon carcinoma cells HCT116 was evaluated using the MTT assay. Chitosan showed the antiproliferative capacity against the colon-cancer-cell HCT116 in a dose dependent manner with IC_{50} of 4.6 mg/ml. Indeed, HCT116 cell proliferation was significantly inhibited ($p < 0.05$) between 13.5 and 67.5% at 0.5–6 mg/mL of chitosan after 24 h of cell treatment. The chitosan showed high antitumor activity which seemed to be dependent on its characteristics such as acetylation degree.

1. Introduction

Oceans constitute an extremely diversified alimentary richness (crustaceans, algae, fish and mollusks). In 2012, more than 158 million of tons of fish and shellfish are actually caught or farmed [1]. Commercial fish production and seafood processing generate large amounts of fish by-products, which create burdensome disposal problems and environmental concerns of such by-products frequently requires additional economic costs. The crustacean processing industry generates a significant amount of by-products, which could represent around 50–70% of the original material. Crustacean by-products are known to be the major source of chitin and chitosan for industrial processing [2,3].

Chitin is a linear polysaccharide consisting of β-(1-4)-*N*-acetyl D-glucosamine residues. It is the second most abundant natural biomass resources derived from exoskeletons of arthropods and from cell walls of fungi [4]. Chemical modification of chitin to generate new functional biopolymers is of interest because the modification would not change the fundamental skeleton of chitin, would increase water-solubility, keep the original physicochemical and biochemical properties and finally would bring new or improved properties [5]. Owing to their versatile biological activity, excellent biocompatibility, and complete biodegradability in combination with low toxicity, both chitin and its modified derivatives have extensive applications in medicine, agriculture, and food industries as well [6]. Usually chemical deacetylation is performed to produce the most common derivative of chitin, named chitosan. Chitosan is a deacetylated form of chitin having D-glucosamine repeating units linked by (1–4) glycosidic bond. It is rigid and specific crystalline structure made it to exist in nature in different polymorphic forms having various properties [7]. Chitosan have great economic impact due to their biological activities and their industrial and biomedical applications. Due to the excellent properties of chitosan such as adsorption [8], film-forming and antimicrobial properties [9,10]. It is used in food preservation [11], cosmetics [12,13], agriculture [14], biotechnology [15], textiles [16,17] as well as medical fields [18].

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http://dx.doi.org/10.1016/j.ijbiomac.2016.02.057
0141-8130/

Microbial contamination in foods not only results a reduction of shelf life and food deterioration, but also leads to disease and economic losses. The growth of microorganisms in food products may cause intestinal disorders, diarrhea and vomiting. Moreover, the increasing antibiotic resistance of some pathogens is a major problem throughout the world [19], and the emergence of antibiotic-resistant microorganisms has decreased the treatment options. Thus, there has been an increased interest in the development of antimicrobial substances from natural products. Chitosan has been of interest in the food industry. It has a track record for its inherent antimicrobial properties against a broad spectrum of organisms [20,21]. Cancer is the second largest non-communicable disease, and it has a sizable contribution in the total number of deaths [22]. The use of naturally occurring compounds with antitumor properties has attracted much interest in chemotherapy and treatment of cancers. In recent years, numerous studies have been reported on the anti-proliferative activity of chitosan [3,23–25].

The aim of this study was to extract chitin from Norway lobster by-products using proteases aided process. The obtained chitin will be characterized and deacetylated to the chitosan. The physicochemical, antibacterial and anti-proliferative characteristics of prepared chitosan were also investigated.

2. Material and methods

2.1. Reagents

Savinase® 16L. tvoe EX serine-protease from *Bacillus lentus* was obtained from Novozymes® (Bagsvaerd, Denmark). The chemicals used in the experimental assays, including 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), cell culture medium (RPMI1640), fetal calf serum (FCS), phosphate buffer saline (PBS), trypsin-EDTA, penicillin and streptomycin mixture and L-glutamine were purchased from GIBCO-BCL (UK). All chemicals and solvents used in the present study were of analytical grade or highest purity available. All solutions were freshly prepared in distilled water.

2.2. Raw material

The Norway lobster (*Nephrops norvegicus*) is one of the most economically important fisheries resources in the North-east Atlantic and Mediterranean areas. Norway lobster by-products (heads, thorax and appendix) were obtained from a local marine processing industry (CALEMBO) in Sfax, Tunisia. By-products were packed in polyethylene bags, placed in ice and transported to the research laboratory. Upon arrival, by-products were washed twice with water, dried and mortar. After drying, they were stored at −20 °C until used.

2.3. Chitin extraction

2.3.1. Demineralization

Demineralization was carried out in HCl medium according to a method developed by Madhavan and Nair [26]. Solid fractions obtained after hydrolysis by Savinase® were treated with HCl (1.5 M) at a ratio of 1:10 (w/v) for 1, 2, 4 and 6 h at room temperature under constant stirring. The chitin residues were filtered, washed to neutrality with deionized water and then drying in a dry heat incubator at 50 °C. Degree of demineralization (DDM) was expressed as a percentage and computed by the following equation [27]:

$$\%DDM = \frac{[(A_O \times O) - (A_R \times R)]}{A_O \times O} \times 100$$

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.

2.3.2. Enzymatic deproteinization

Demineralized samples were mixed with water at a ratio of 1:3 (w/v), minced then cooked for 20 min at 90 °C. The cooked sample was then homogenized in a Moulinex R62 homogenizer (Organotechnie, Courneuve, France) for about 5 min. The pH and temperature of the mixture were adjusted to 10.0 and 55 °C, respectively. Then, the sample proteins were digested with Savinase® using different E/S ratio (Units of enzyme/mg of protein). Protein content was determined by kjedahl method. After incubation for 4 h at 55 °C, the reaction was stopped by heating the solution at 90 °C during 15 min to inactivate the enzyme. The solid phase was washed and then pressed manually through four layers of gauze and then drying at 50 °C. Deproteinization (DP) was expressed as percentage and computed by the following equation as described by Rao et al. [27].

$$DP(\%) = \frac{(P_O \times O) - (P_R \times R)}{P_O \times O} \times 100$$

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

2.4. Chitosan preparation

The chitin was treated with 50% (w/v) NaOH at 120 °C for 4 h until it was deacetylated to chitosan perfectly soluble in might acidic conditions. After filtration, the residue was washed with distilled water and the crude chitosan was obtained by drying in a dry heat incubator at 50 °C overnight.

2.5. Physicochemical characterization

2.5.1. Chemical composition

Moisture and ash contents were determined according to the AOAC standard methods 930.15 and 942.05, respectively [28]. Protein contents and crude chitin were determined by extracting samples with 10% (w/v) NaOH for 20 min at 120 °C. The supernatant was retained by filtration and diluted to 100 ml with distilled water. This extract was used for protein determination (total nitrogen contents × 6.25) according to the Kjeldahl procedure. Crude fat was determined gravimetrically after the Soxhlet extraction of dried samples with hexane. Water activity (a_w) was measured at 25 °C by a NOVASINA aw Sprint TH-500 apparatus (Novasina, Pfäffikon, Switzerland). All measurements were performed in triplicates.

2.5.2. Color

The samples were placed between two steel dishes with a hole of 5.7 cm diameter. The color was determined with a tristimulus colorimeter (CHROMA METER CR-400/410. KONICA MINOLTA, Japan) using the CIE Lab scale (C/2°), where L^*, a^* and b^* refer to the parameters measuring lightness, redness, and yellowness, respectively. A standard white plate was used as a reference. The results were the average of five measurements taken at ambient temperature and at different points on the samples.

2.5.3. Infra-red spectroscopic and X-ray diffraction analysis

The absorption spectra of the samples were obtained using FTIR spectroscopy (Analect Instruments fx-6 160). The sample was thoroughly mixed with KBr, the dried mixture was then pressed to result in a homogeneous sample/KBr disc. The FTIR spectra of the prepared materials were recorded between 400 and 4000 cm^{-1} in