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Synthesis, Characterization and Antioxidant Activities of Bioactive Polymer from Internal Bone of *Sepia aculeata*

ASWIN GOWRI PRASAD[1], YAGNIYASREE MANOGARAN[2,3] and PASIYAPPAZHAM RAMASAMY[2,3,*]

[1]Department of Physiology, Saveetha Dental College & Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai-600077, India
[2]Polymer Research Laboratory, Centre for Marine and Aquatic Research (CMAR), Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai-602105, India
[3]Department of Prosthodontics and Implantology, Saveetha Dental College & Hospitals, Saveetha Institute of Medical and Technical Sciences (SIMATS), Saveetha University, Chennai-600077, India

*Corresponding author: E mail: ramkanth281@gmail.com

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To study the antioxidant potential of chitosan by different *in vitro* methods, including chelating ability on metal ions, DPPH and superoxide radical assays. Chitin was extracted from internal bone of *Sepia aculeata* (cuttlebone) and through deacetylation, chitosan was prepared. The extracted chitosan was characterized with FT-IR, FESEM, XRD and exhibits a crystalline, non-porous and smooth membranous structure. The antioxidant activity was performed to determine if the isolated bioactive compounds exhibit a potency against the free radicals. Furthermore, chitosan demonstrated antioxidant activity against DPPH at 14.08–51.17% at 0.1–10 mg/mL and superoxide radicals at 24.38–67.19% at 0.1–1.6 mg/mL. At the same time, compared to EDTA, ferrous ions showed a poor chelating activity of 28.20-74.60% at concentrations of 0.1–10 mg/mL. These results indicated that cuttlebone, due to its superior antioxidant activity and ferrous ion chelating ability, can serve as an effective organic antioxidant and food additive in the pharmaceutical industry.

Keywords: Cuttlefish, DPPH, Chitosan, Bioactive polymer, Superoxide radical, Antioxidant activity.

INTRODUCTION

Humanity and the sea have always been associated with travel, trade and food production. Despite covering more than 70% of the earth's surface, the oceans contain 95% of biosphere [1]. The second most common biopolymer in nature is chitin, which is present in the cuticles of insects, the cell walls of fungus and the shells of crustaceans [2]. Chitin is a straight chain polymer with three different structural classes *viz.* α-, β- and γ-chitin and made up of β-1,4-N-acetylglucosamine [3,4].

The straight-chain polymers glucosamine and N-acetyl glucosamine, which make up chitosan, can also partially deacetylate chitin [5]. The most prevalent chitin in nature is called α-chitin, which is found in crabs, shrimp and lobster. On the other hand, β-chitin, which is found in squid, possesses intrasheet hydrogen bonds formed by parallel chains [6,7]. But γ-chitin, which is a blend of α- and β-chitin, is present in the cell walls of fungi and has a features of mixed parallel and anti-parallel chains [6]. Several studies have explored the medical applications of chitin and chitosan due to their numerous beneficial biological properties, including their biocompatibility, ability to break down, halt bleeding and help in wound healing [8].

Due to these unique qualities, chitosan and its derivatives have found applications in the health care, food, agricultural, biological sciences and medicinal sectors [9-11]. Several researchers have also thoroughly studied the antioxidant qualities of chitosan [11-14]. Nonetheless, the information regarding the antioxidant properties of chitosan derived from crab shells is insufficient. The aim of the work was to extract the bioactive polymer (chitosan) from the internal bone of *Sepia aculeata* (cuttlebone) and characterized using FT-IR, FESEM and XRD in order to ascertain its structure and also evaluated the *in vitro* free radical chains reactions.

EXPERIMENTAL

The analytical grade chemicals *viz.* butylated hydroxyanisole (BHA), EDTA, potassium ferricyanide, linoleic acid, ascorbic acid, butylated hydroxyanisole (BHA), ferrozine, potassium permanganate were purchased from Sigma-Chemical Co., USA. The other reagents like $FeCl_2$ and H_2O_2 were procured from Merck Ltd., India.

Extraction of chitin from internal bone of *Sepia aculeata*: To extract chitin from the internal bone of *S. aculeata*, the calcification and protein separation techniques were applied as per reported method [15]. The mass of dried chitin sample was measured and the content of the chitin was calculated. Neutral pH was maintained at every step to obtain pure sample.

Synthesis of chitosan from extracted chitin: The deacetylation process was used to transform the obtained chitin into chitosan as reported by Younes & Rinaudo [16].

Characterization: Powdered samples of chitin and chitosan were used to prepare KBr dics. The FTIR spectra were recorded on a FTIR spectrometer (BRUKER ALPHA II FTIR spectrum analyser). The microstructure and surface morphology of chitosan were studied using a Hitachi Hus-4 vacuum evaporator coated with a thin layer of gold/palladium. The alloy was evaporated directly onto the sample at 20 V for the FESEM analysis. The X-ray diffractogram was acquired utilizing a Bruker X-ray diffractometer (D2 PHASER, Bruker AXS Inc.) operating at 40 kV and 30 mA with Cu$K\alpha$ radiation, scanned at a rate of 2° min^{-1} across 2θ angles ranging from 5° to 90°.

in vitro Free radical chain reaction of chitosan

DPPH free radical scavenging activity: The solutions were prepared using various concentrations, ranging from 0.1 to 10 mg/mL. After dissolving in 4 mL of 2 g/L acetic acid solution, it was mixed followed by the addition of 1 mL of DPPH radical containing methanolic solution to obtain a 10 mM/L of DPPH solution. The mixture was well shaken and then allowed to ideal in the subdued light for 0.5 h. A UV/vis spectrophotometer was used to measure the absorbance at 517 nm [17,18]. BHA and ascorbic acid were used as controls. The scavenging efficiency was estimated according to eqn. 1:

$$\text{Scavenging efficiency } (\%) = \frac{\Delta A_{control_{517}} - \Delta A_{sample_{517}}}{\Delta A_{control_{517}}} \times 100$$

Superoxide radical scavenging activity: The sample was mixed at concentrations ranging from 0.005-0.4 mg/mL with 0.1 M of phosphate buffer at pH 7.4, PMS (30 mM), NBT (72 mM) and NADH (338 mM). The mixture was allowed to incubate at room temperature for a period of 5 min. The absorbance was measured at 560 nm using a UV/vis spectrophotometer against a blank [18,19]. The superoxide radical scavenging capacity was determined using eqn. 2.

$$\text{Scavenging efficiency } (\%) = \frac{\Delta A_{control_{560}} - \Delta A_{sample_{560}}}{\Delta A_{control_{560}}} \times 100$$

Chelating activity on ferrous ions: The ferrous ion binding capacity was estimated as per reported method [18,20]. When ferrocine and ferrous ions combine, a crimson colour is formed. The formation of the complex is inhibited by chelating compounds, which diminishes the development of crimson colour. The colour reduction measurement is used to determine the binding capacity of chelator. In brief, 1 mL of acetic acid, at different concentration varied from 0.1 to 10 mg/mL, was applied to each sample. Subsequently, it was combined with 0.1 mL of 2 mM/L $FeCl_2$ and 3.7 mL of methanol. To the process, 0.2 mL of 5 mM/L ferrozine was added. A UV/vis spectrophotometer was used to determine the absorbance at 562 nm relative (which is due to the ferrous ion-ferrozine complex) to a blank. The weaker the ferrous iron binding strength is correlated with higher chelating power.

RESULTS AND DISCUSSION

The yield percentages of chitin and chitosan were found to be 37% and 84%, respectively. By deacetylating chitin, usually with alkaline hydrolysis, chitosan is obtained. Different species produce different amounts of chitin. For example, *Sepia officinalis* cuttlebone had a yield of 20% [21]; *Loligo lessoniana* had a yield of 36.06%; *Loligo formosana* had a yield of 36.55% [22] and *Penaeus monodon* had a yield of 22.18% [23]. In the current study, 37% of chitin was extracted from the cuttlebone of *Sepia aculeata*. Unfortunately, chitin, a polymer, is resistant to water (hydrophobic), limiting its potential uses, therefore, derivatives of chitin, such as chitosan, is considered as a better choice to improve its solubility and uses. Researchers found 35.43% chitosan output from the operculum and shell of *Nerita crepidularia* and 33.02% from the squid *Doryteuthis sibogae* gladius [24]. The deacetylated version of chitin and chitosan, serves as a foundation for the additional modifications. The production percentage of chitosan was, in contrast, higher than that of *S. pharaonis* and *Donax scortum* [25].

FTIR studies: Fig. 1 shows that the broad band at 3364 cm^{-1} was due to the hydroxyl stretching vibration of the polysaccharide. A large peak preent in the 3500-1500 cm^{-1} range indicates that few hydrogen bonding when hydroxyl chains are present. The weak absorption bands were caused by CH_2 groups' C-H stretching vibrations and the stretching forces of the CHO and C=O bonds were responsible for the absorptions at 1629-1561 cm^{-1}. The deforming vibrations of C-H bond were caused due to the strong broad absorption bands in the 3364-1410 cm^{-1} region. The polysaccharide absorbance ranged from 1100-570 cm^{-1} corresponds to the sites of the C-O and C-C link bands.

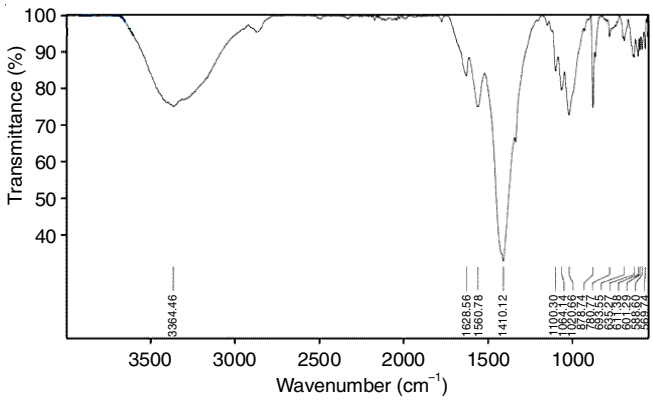

Fig. 1. FT-IR spectral analysis of *Sepia aculeata* chitosan

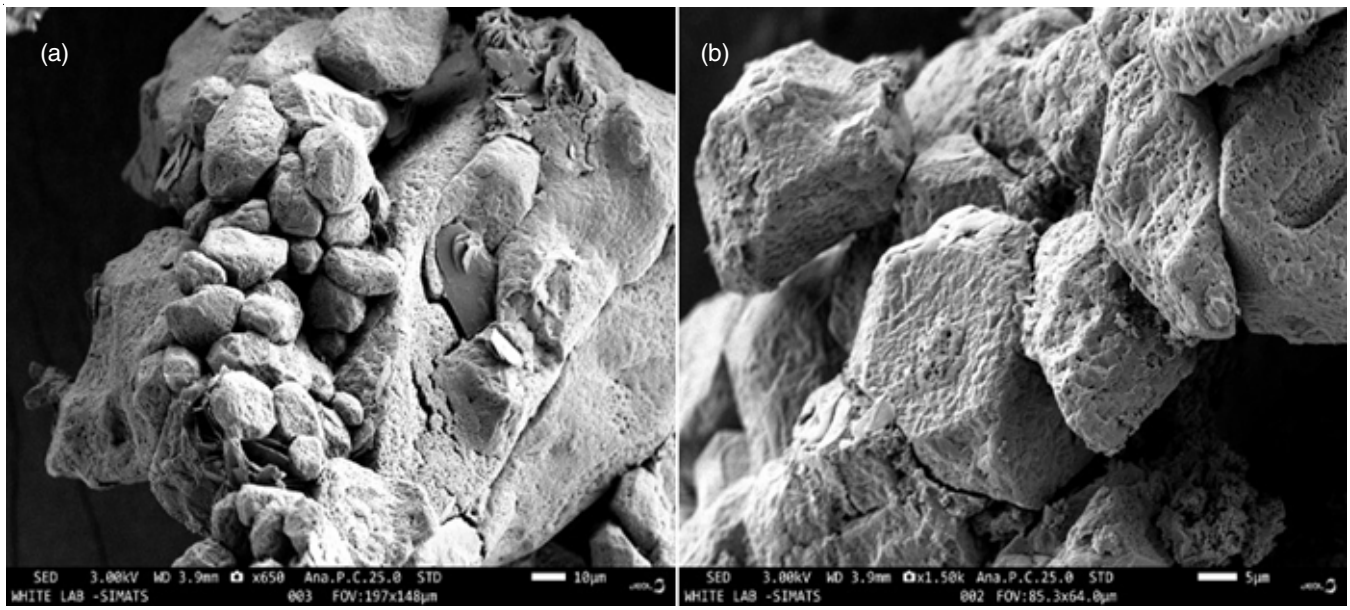

Fig. 2. FESEM images of *Sepia aculeata* chitosan

In the FTIR spectrum of chitosan, the characteristic peaks associated with their functional groups are appeared. For example, a peak at 3500-3400 cm^{-1} indicates an amino group (-NH_2), signifying the stretching vibration of N–H bonds. The carbonyl stretching vibration (C=O) of amide group (-CONH) appears as narrow band at the 1655-1650 cm^{-1} region [26]. The stretching vibrations of the C-O-C glycosidic linkages appear at 1150-1050 cm^{-1} in the polymeric backbone of chitosan. Moreover, the 1150-890 cm^{-1} area reflects the C-O and C-N bond bending vibrations, offering the additional structural insights into chitosan [27].

Morphological studies: The FESEM images (Fig. 2a-b) depict the surface morphology of chitosan, displaying a smooth membranous phase characterized by a non-porous structure composed of crystallites, dome-like openings and microfibroid hollows that also reveal a dome shape. In contrast, microfibrils modify their shape and surface roughness in response to their presence.

XRD studies: Two prominent broad and strong peaks at 2θ = 30° and 37° and also few weak peaks at 11°, 19°, 33° and 34° were observed (Fig. 3). The results of the prepared chitosan are similar to those reported by Kasongo *et al*. [28], who observed the clear and distinct reflections of chitosan derived from mollusc chitin.

In vitro antioxidant activity of chitosan

DPPH radical scavenging activity: The DPPH assay was employed to evaluate ability of the chitosan to scavenge free radicals. In the current investigation, chitosan demonstrated a scavenging ability of 14.08-51.17% at 0.1-10 mg/mL. Nonetheless, at 0.1-10 mg/mL, ascorbic acid demonstrated scavenging abilities at a level of 26.36-71.78% (Fig. 4a).

Superoxide radical scavenging activity: The superoxide radicals produced from dissolved oxygen through PMS-NADH coupling can be measured by their ability to reduce NBT. In this work, chitosan can scavenge superoxide radicals at the

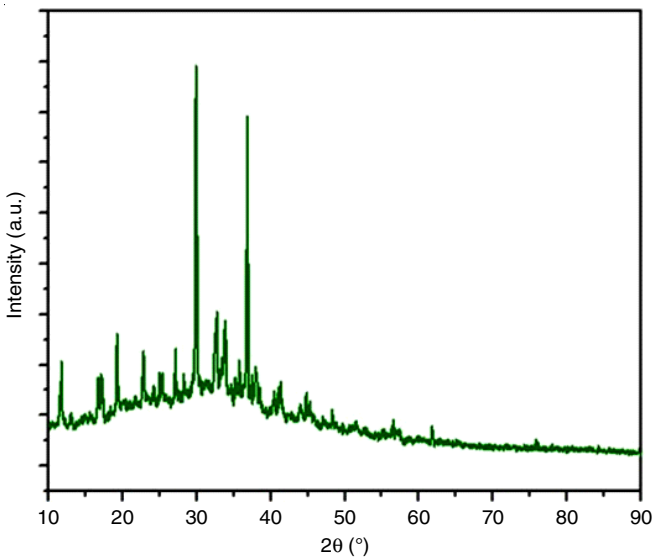

Fig. 3. XRD spectrum of *Sepia aculeata* chitosan

concentrations between 24.38 and 67.19% at 0.1-1.6 mg/mL. However, α-tocopherol display the scavenging ability of about 31.11-77.46% at 0.1-1.6 mg/mL concentrations (Fig. 4b).

Chelating activity on ferrous ions: In this study, it was found that chitosan could bind to Fe^{2+} ions with a strength ranging from 28.20% to 74.60% at concentrations of 0.1 mg/mL to 10 mg/mL. Nevertheless, at 0.1–10 mg/mL, EDTA showed a 42.22–97.92% chelating capacity (Fig. 4c). Unlike chitosan, EDTA exhibited a more potent chelating effect owing to its capacity to develop hexadentate complexes with lesser entropic fluctuations compared to the chelation of a single ferric ion.

Conclusion

The chitosan was extracted from the internal bones of *Sepia aculeata* (cuttlebone) and characterized. The antioxidant activity of chitosan and the iron binding ability were also eval-

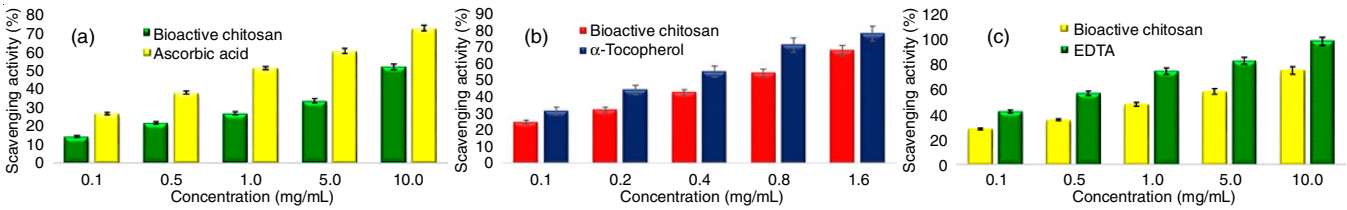

Fig. 4. (a) Scavenging activity of DPPH radicals, (b) scavenging activity superoxide radical and (c) chelating ability on ferrous ions

uated. The DPPH and superoxide radical scavenging assays demonstrated the high antioxidant and free radical scavenging activities. It also chelates iron and has reducing power. Further investigation into chitosan's antioxidant mechanisms and its physiological interactions may open the door to new therapeutic modalities.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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