Middle-East Journal of Scientific Research 16 (10): 1446-1451, 2013
ISSN 1990-9233
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DOI: 10.5829/idosi.mejsr.2013.16.10.12033

Antioxidant Activity of the Chitosan Extracted from Shrimp Exoskeleton

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Abstract: The chitosan were isolated from shrimp exoskeleton. The Extracted chitosan have antioxidant and cytotoxic potentials were investigated byemploying various analysis such as 1,1-diphenyl-2-picrylhydrazyl (DPPH)/superoxide/ hydroxyl radicals scavenging, reducing power, iron ion chelating and total antioxidant activity. The extracted chitosan have potentialantifungal and cytotoxic activity were studiedat various concentrations.

Key words: Chitosan · Antioxidant · Shrimp shell · Antifungal activity

INTRODUCTION

Antioxidant activity is one of the well-known functions of chitosan. Many studies have shown that chitosan inhibit the reactive oxygen species (ROS) and prevent the lipid oxidation in food and biological systems. Several mechanisms about the antioxidant action of chitosan have been proposed [1]. Chitosan can scavenge free radicals or chelate metal ions from the donation of a hydrogen or the lone pairs of electrons [2] [3]. The interaction of chitosan with metal ions could involve several complex actions including adsorption, ion-exchange and chelation [4]. The hydroxyl groups (OH) and amino groups (NH_2) in chitosan are the key functional groups for its antioxidant activity, but can be difficult to be dissociated due to the semi-crystalline structure of chitosan with strong hydrogen bonds [2].

Chitosan, a cationic polysaccharide with a high molecular weight, linear polymer that comprises β-1,4-linked glucosamine (GlcN) with various amounts of N-acetylated GlcN residues. It is typically obtained by the alkaline deactivation of chitin extracted from an abundant source of shellfish exoskeletons or the cell walls of some microorganisms and fungi [5]. It has attracted marked interest as a biomedical material, because it has unique biological activities, such as antitumor [6], immune stimulatory [7] and antibacterial [8], [9] activities.

Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various degenerative diseases as well as in the normal process of aging.Reactive oxygen species (ROS) in the forms of superoxide anion, hydroxyl radical and hydrogen peroxide (H_2O_2) are generated by normal metabolic process or from exogenous factors and agents and they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. These ROS are capable of damaging a wide range of essential biomolecules.

In recent years, various investigators have observed the antioxidant activity of chitosan derivatives. There has been increasing interest in finding natural antioxidants, since they can protect the human body from free radicals and retard the progress of many chronic diseases.[10].In general, the natural antioxidants mainly constitute a broad range of compounds including phenolic compounds, nitrogen compounds and carotenoids [11] However, the antioxidant activities of several biological polysaccharides have recently been described. The antioxidant activity of chitosan and its derivatives also has attracted the most attention[12]. In this study, we studied the antioxidant activities of chitosan, by employing various assays, such as DPPH/superoxide/hydroxyl radicals scavenging, iron ion chelating and so on, in order to understand the mechanisms of their antioxidant activities and cytotoxic studies.

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MATERIALS AND METHODS

Collection of Shrimp Shell: Shrimp shell were collected from local market, Madurai, India and then processed, washed, sun dried and cut into pieces for utilization to extract chitin and chitosan from it.

Extraction of Chitin from Shrimp Shell: For protein removal from shrimp shell, 40% NaOH (w/v) was added into shrimp shell and the deproteination process was led for 72 hours. After deproteination by NaOH, the shells were washed with water. Then these shells were treated with 4% concentrated HCl in order to remove the calcium from shells and the demineralization time was 20-24 hours and then washed with water and dried. Thereafter, deproteinated and decalcified shells were the chitin.

Conversion of Chitin to Chitosan: To produce chitosan, dried deproteinated and decalcified chitins were moisturized with water. For deacetylation, then moisturized chitin was put into 20 M NaOH solution and stirred. Deacetylation process was carried out for 48 hours. After deactivation, chitosan flakes were washed, squeezed and dried in a forced air oven at 60-70°C.Chitosan were extracted from shrimp shell waste and the obtained chitosan are used for further antioxidant assays.

Antioxidant Determination Assay

DPPH Radical Scavenging Assay: The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavengingactivity of the chitosan at a concentration of 1 mg mL)1 was determined as described by [13]. Samples dissolved in 500 lL of distilled water (1 mg mL) were mixed with 375 lL of 99.5% ethanol and 125 lL of 0.02% DPPH in 99.5% ethanol. The mixtures were then incubated for 60 min in the dark at room temperature and the reduction of DPPH radical was measured at 517 nm using an UV-visible spectrophotometer. In its radical form, DPPH has an absorption band at 517 nm, which disappears upon reduction by an antiradical compound. Lower absorbance of the reaction mixture indicated higher DPPH-free radical-scavenging activity. DPPH radical-scavenging activity was calculated as follows:

$$\text{Radical-scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The control was conducted in the same manner,except that distilled water was used instead of sample.BHA was used as positive standard. The test was carriedout in triplicate.

Super Oxide Radical Scavenging Assay: The superoxide scavenging ability of chitosan was assessed by the method [14]. The reaction mixture, containing sulfated chitosan (0.05 - 0.5 mg/mL), PMS (30 µM), NADH (338 µM) and NBT (72 µM) in phosphate buffer (0.1 M pH 7.4) was incubated at room temperature for 5 min and the absorbance was read at 560 nm against a blank. The capability of scavenging the superoxide radical was calculated using the following equation

$$\text{Scavenging effect (\%)} = (1 - A_{\text{sample 560 nm}}) / A_{\text{control 560 nm}}$$

Hydroxyl Radical Scavenging Assay: The hydroxyl radical scavenging assay was assessed by the method[15]reaction mixture containing chitosan (0.1 - 3.2mg/mL), was incubated with deoxyribose (3.75 mM), H2O2 (1 mM), FeCl3) (100 µM), EDTA (100 µM) and ascorbic acid (100 µM) in potassium phosphate buffer (20 mM, pH 7.4) for 60 min at 37. The reaction was terminated by adding 1ml of TBA (1%, w/v) and 1ml of TCA (2%, w/v) and then heating the tubes in a boiling water bath for 15 min. The contents were cooled and the absorbance of the mixture was measured at 535 nm against reagent blank. Decreased absorbance of the reaction mixture indicated decreased oxidation of deoxyribose.

Measurement of Reducing Power: The reducing power of chitosan was quantified by the method [16]. Briefly, reaction mixture 1 mL, containing different concentration of chitosan in phosphate buffer (0.2 M, pH 6.6), was incubated with potassium ferricyanide (1%, w/v) at 50 for 20 min. The reaction was terminated by adding TCA solution (10%. w/v) and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was mixed with distilled water and ferric chloride (0.1%, w/v) solution and the absorbance of the reaction measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Metal Ion Chelating Assay: The ferrous ion-chelating potent Antioxidant activity assessed by the method [17-20]. The inhibitory effect of chitosan scavenging activity of superoxide radicals was marked and concentration related. A significant scavenging effect

(20.4 - 88.6%) of super radicals was evident at all tested concentrations of chitosan was investigated according to the method of wherein the Fe2+ -chelating ability of chitosan was monitored by measuring the ferrous iron-ferrozine complex at 562 nm. Briefly, the reaction mixture, containing chitosan of different concentrations, FeCl2 (2 mM) and ferrozine (5 mM), was adjusted to a total volume of 0.8 mL with water, shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against blank. EDTA was used as a positive control. The ability of chitosan to chelate ferrous ion was calculated using the following equation:

Chelating effect (%) = (1 - Asample 562 nm) / Acontrol 562 nm

Antifungal Activity: Antifungal activity of chitosan was screened by agar tube dilution method. *Aspergillusniger* and *Aspergillusoryzae*were used as fungal strains. Fungus media was prepared by dissolving 6.5g of sabouraud dextrose agar (MERCK) in 100 ml of distilled water and pH was adjusted as 5.6. Agar was poured in screwed capped test tubes and was autoclaved. Agar containing test tubes were allowed to cool up to 50°C, added test sample in each tube and allowed to solidify in slanting position. After solidification, each slant was inoculated by 4 mm diameter piece of fungal strain.The positive and negative control is also used. These test tubes were incubated at 28°C for seven days. Readings were measured in mm and growth inhibition was calculated with reference to negative control. Percentage inhibition was calculated.

***In vitro* Cytotoxicity Studies**

MTT Dye Reduction Test:

- Grow 10-ml yeast cultures over night to saturation in appropriate media at 30°C.
- Spin cells down for 5 min at 1,500 rpm (Beckmann) and resuspend the cells in 0.5 ml sterile distilled water.
- 50µl of MTT dye and 100µl of extracted chitosan was added.
- Incubate for 37°C for 3 hours. (Cover with Aluminium foil because MTT is light sensitivity)
- Added to 200µl of PBS to the tubes.
- Spin the 10,000 rpm for 3 minutes after 30 minutes incubation.

Decant the supernatant and suspend in 200µl of n-propanol in 0.04N HCl.

- And kept in a dark place for overnight and read at 650nm.

Result: Figure 1 shows the various steps involved in the chitosan preparation from raw material shrimp exoskeleton, where major steps such as deproteination, demineralization, decolourisationand deacetylation.

The extracted chitosan have potent antioxidant activity. Figure 2 shows the total antioxidant scavenging potential of DPPH where BHA and ascorbic acid, used as the positive scavenger and measured at various concentrations. The chitosan was found to be equally good in the DPPH radical-scavenging activities.

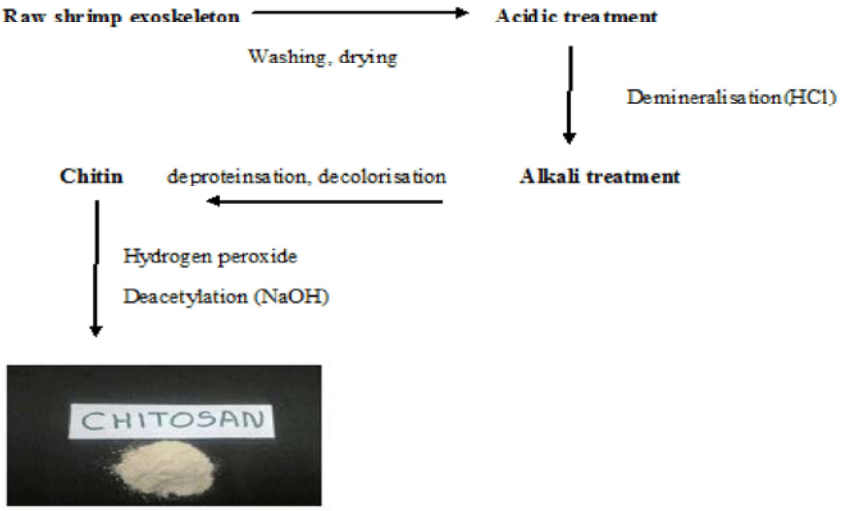

Fig. 1: Extraction of chitosan from shrimp exoskeleton

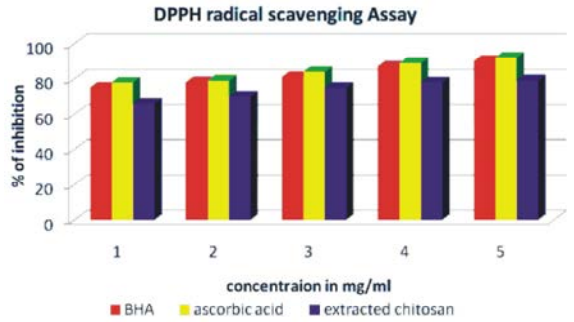

Fig. 2: DPPH radical scavenging Assay

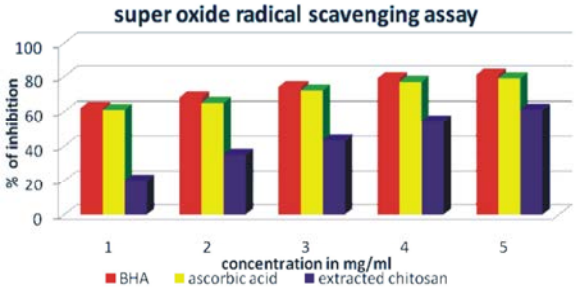

Fig. 3: Super oxide radical scavenging assay

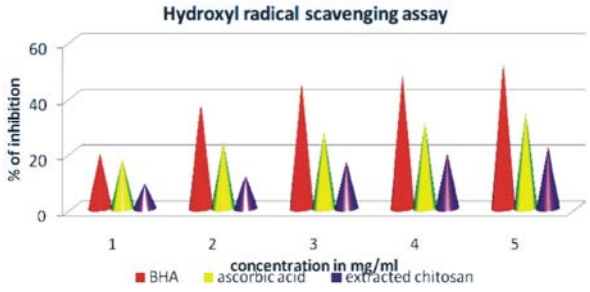

Fig. 4: Hydroxyl radical scavenging assay

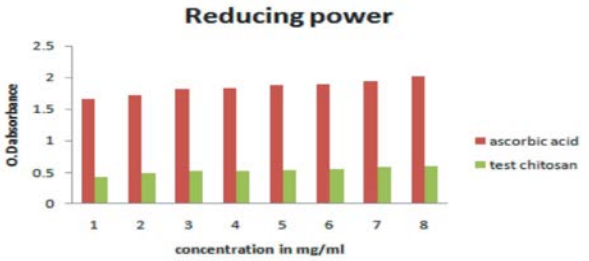

Fig. 5: Reducing power activity of extracted chitosan

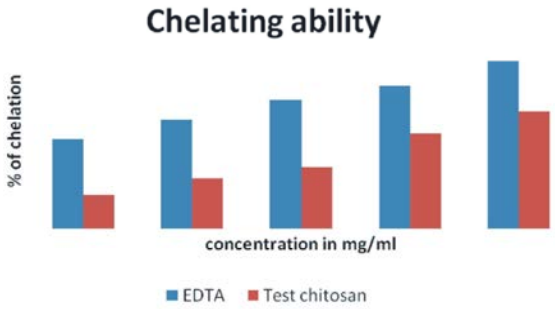

Fig. 6: Chelating ability of extracted chitosan

When compared to the positive controls the radical scavenging activity was seen in the order followed by the BHA, Ascorbic acid and chitosan. The figure 3 shows inhibitory effect of chitosan such as scavenging activity of superoxide radicals at various concentration. A significant scavenging effect of super oxide radicals was evident at all tested concentrations of extracted chitosan and compared with standard of BHA and Ascorbic acid.The Figure 4shows the effect of chitosan, BHA and ascorbic acid on hydrogen peroxide scavenging activities.

Table 1: Antifungal activity of extracted chitosan against various pathogens

	Concentration of chitosan in µg/ml				
Name of the Pathogens					
Zone of inhibition in mm	10	20	30	40	50
Antibiotic control					
Fluconazole	11	13	14	15	17
Aspergilusniger	9	10	11	13	15
Aspergillusoryzae	10	12	13	15	16

Table 2: Cytotoxic activity of extracted chitosan at various concentration in yeast cells

Concentration of extracted chitosan(µg/ml)	Control % of cytotoxic effect	Extracted Chitosan % of cytotoxic effect
10	100	90
20	100	85
30	100	72
40	100	69
50	100	61

Scavenging activity of hydrogen peroxide, at various concentration shows the oxidative damage, induced by Fe^{3+}/ H_2O_2 on deoxyribose, is plotted in figure 4. Nearly 20% of inhibition was observed at the highest concentration, 55 and 35% was observed in Ascorbic acid and BHA respectively.The reducing power of chitosan was increased with the increasing concentration. Figure 5 shows the reducing power increased with increasingconcentrations.The ferrous ion-chelating effect of chitosan was concentrated related as shown in Figure 6. At 1 mg/mL, chelating ability of chitosan on ferrous ions was 62.6%. However, EDTA showed a 68% chelating ability.

Table 1 shows that the extracted chitosan have potential antifungal activity. The extracted chitosan at different concentration shows zone of inhibition. Extracted chitosan have good antifungal activity when compared with standard drug of Fluconazole. The *in vitro* cytotoxicity assay was assessed by using MTT dye reduction method in yeast cells. The Table 2 shows Cytotoxic activity of extracted chitosan at various concentration in yeast cells.

RESULT AND DISCUSSION

This investigation suggest that the antioxidant property of extracted chitosan in Pharmaceutical industry is in need of different types of chitosan presently available in the market which are to be refined further more to meet the required standards. For drug delivery, special preparation techniques are used to prepare chitosan drug carriers by taking care such parameters as cross linker concentration, chitosan molecules weight and processing conditions all these effect release rate of the loaded drug. Chitosan seems to be the biopoly-mer for the development of new derivatives such as Water-soluble derivatives of chitosan, Quaternarized derivatives, Caboxyalkylation, Chitosan Esters, sulfonated derivatives of chitosan, N-trimethylene chloride chitosan, Chitosan conjugates.

CONCLUSION

The results of this work demonstrate the extracted chitosan exhibit potent antioxidant activity and free radical scavenging activity, including activity toward DPPH radicals, hydrogen peroxide and superoxide anion radicals. They also exhibit ferrous ion-chelating activity toward ABTS radicals. Theassays were useful in establishing the antioxidant capacities of chitosan, which have important applications in food industries. However, *in vivo* antioxidant activity and the various antioxidant mechanisms must be investigated further.

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