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PHYSICAL-CHEMICAL CHARACTERIZATION AND BIOLOGICAL EVALUATION OF CHITOSAN EXTRACTED FROM MARINE WASTE

Carla-Cezarina PĂDUREȚU[1], Manuela Rossemary APETROAEI[2], Nicoleta BADEA[1], Laura BUCUR[3], Ileana RĂU[1], Verginica SCHRÖDER[3]

[1]University Politehnica of Bucharest, Faculty of Applied Chemistry and Materials Science, 1-5 Polizu Str.,060021, Bucharest, România
[2"]Mircea cel Batran" Naval Academy, Faculty of Navigation and Naval Management, 1 Fulgerului Str., 900218, Constanta, Romania
[3]Ovidius University of Constanta, Faculty of Pharmacy, 1 University Str., Campus Corp C, Constanta, Romania

Corresponding author email: verginica.schroder@univ-ovidius.ro

Abstract

Chitosan is a well known biopolymer with applications in various areas, and especially in pharmaceutical and medical fields. The main source of chitosan is represented by the marine waste. The present study was focused on chitosan extraction from shrimp waste using a classical chemical procedure. The obtained chitosan was characterized regarding its physical-chemical properties,and also the antioxidant activity and hemolytic activity were evaluated. In this study, the chitosan samples were dissolved in diluted solutions of acetic acid and lactic acid. The results showed that the best antioxidant activity was obtained for the chitosan dissolved in acetic acid. Also, the chitosan samples produced significant effects on the red blood cells activity. All these results suggest that new potential applications could be envisaged.

Key words*: antioxidant activity, biological evaluation, chitosan, hemolytic activity.*

INTRODUCTION

The marine waste is a source of pollution because of its perisability and its high pollution effect if discarded off-shore (Morganti, 2013). Thus, the waste has valuable components such as proteins, salts and chitin that could be recovered for further uses (Dima et al., 2017). The most well-known derivative of chitin ischitosan, which presents biocompatible and biodegradable properties andis obtained by *N*-deacetylation of chitin (Sagheer et al., 2009; Mohanasrinivasan et al., 2014). Chitosan is used in many biomedical applications due to its biocompatibility capacity (Shigemasaand Minami, 1996) and the research carried out over the time exhibited good results of chitosan in treating wound shealing (Alsarra, 2009; Wiegand et al., 2010) or its use as tissue adhesives (Barton et al., 2014). Regarding the chemical structure of chitosan, although the active hydroxylandamino groups in the polymerchains represent the origin of its scavenging capacity, also the deacetylation degree and the molar mass affect the antioxidant activity. Chitosan with very low deacetylation degree has little chelating activity while chitosan with high molecular mass has less scavenging activity because of the strong intramolecular hydrogen bonding that act restrictive to the oxidative agents exposure (Alishahi, 2012).

Chitosan may also behave as hemostatic agent, as previously reported (Nuntanaranont et al., 2018; You, 2016). The hemostatic mechanism of chitosan involves the agglutination of red blood cells, mainly because chitosan is a positively charged polymer that attracts the negatively-charged red blood cells to agglutinate and promote clotting (Zhou et al., 2017).

The main purpose of this study was to extract chitosan using shrimp waste, to evaluate its physical-chemical properties and also the ability to capture long and short term life radicals using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) assay, respectively chemiluminiscence method. Furthemore, the hemolytic activity of the studied chitosan samples was also tested for

identification of biological effect with different further applications.

MATERIALS AND METHODS

Characterization of raw material

Marine waste was used in this study and the samples consisted in exoskeleton fragments from *Palaemon elegans* (Rathke, 1837) species, which is also known as the rock shrimp (Bacescu, 1967). The samples were collected as waste from the seafood restaurants situated on the Romanian Black Sea littoral zone (Figure 1).

Figure 1. Shrimp waste used as raw material

Extraction of chitosan

For the chitosan extraction it wasused a traditional process involving chemical methods (Montilla et al., 2014). Raw material was dried using an oven and was converted into powder using a grinding machine. The obtained powder subjected to demineralization treatment and was performed by immersing the shrimps powder in a solution of 4% HCl (1:13, w/v) for 50 min at room temperature (25°C), while for deproteinization a NaOH solution of 5% (1:20, w/v) for 2 h at 65°C was used. The obtained material was washed once with acetone and rinsed for three times with bidistilled water. Chitindeacetylation was conducted in NaOH solution of 40% (1:15, w/v), for 1 h at room temperature and then 1 h at 95°C. The deacetylation process was repeated using the same experimental conditions in order to obtain a material with less impurities and a higher deacetylation degree. After each chemical treatment the obtained powder was washed with bidistilled water until neutral *p*H was reached, then dried until constant mass.

Characterization of chitosan

Physical-chemical characterization of the obtained chitosan was realized in terms of deacetylation degree, viscosity and ash content. The deacetylation degree was determined using the potentiometric titration method (Dima et al., 2017). The viscosity was determined using a mixture of 2% acetic acid solution and KCl 0.1 M as solvent for chitosan (Sagheer et al., 2009), the measurements were performed at 25 ± 1°C using an Ostwald viscometer, and the data was calculated using the Mark-Houwink equation (Hossain and Iqbal, 2014;Moura et al., 2011). The ash content analysis was performed using a furnace from Caloris Group S.A., model 1206M, L Microterm instrument type and was determined according to F2103–01 standard (2006). For chitosan extraction and characterization, hydrochloric acid, puriss. p.a., and acetone, puriss. p.a., from Sigma-Aldrich, lactic acid from Mayam (p.a. 80%, ρ = 1.15 g/cm^3), sodium hydroxide pellets, glacial acetic acid and potassium chloride from ChimReactiv S.R.L. were used.

Biological evaluation of chitosan

The antioxidant activity of chitosan was tested using two different methods: ABTS and chemiluminiscence. The $ABTS^{\bullet+}$ radical cation solution was prepared according to Rasti et al. (2017) and the sample solutions were prepared using a modified procedure. Volumes of 2 mL $ABTS^{\bullet+}$ and 2 mL of each chitosan sample (0.5-4 g/L) previously dissolved in 1% acetic acid/lactic acid were used. The measurements were realized against a blank sample prepared with 2 mL $ABTS^{\bullet+}$ solution and 2 mL of 1% acetic/lactic acid. Absorbance was measured at 734 nm at time intervals of 4 min using an UV-Vis-NIR Spectrophotometer instrument from Jasco, V-570 type. The analyses were performed in duplicate.

For chemiluminiscence, a Chemiluminometer Turner Design TD 20/20 (USA) instrument was used to scavenge the free oxygen radicals. A system solution containing luminol, Tris-HCl, H_2O_2 and 1% acetic acid/lactic acid was used for the preparation of the blank solution. The samples were prepared similarly, using chitosan dissolved in 1% acetic acid/lactic acid. The tests regarding the hemolytic activity are used to evaluate damage to erythrocytes in the

form of membrane damage as a consequence of physical and chemical interactions with the environment (Hasirci and Hasirci, 2018). The hemolysis assay was realized using a modified method from Hasirci and Hasirci (2018). The blood was collected on EDTA to prevent coagulation, from healthy volunteers with their agreement. The blood sample was centrifuged for 5 minutes at 4000 rpm and the plasma was carefully removed using a micropipette. The erythrocytes were washed several times with saline solution (NaCl 0.9%) until the supernatant became clear. The erytrocitar sediment was diluted with 10x phosphate buffer saline (PBS) in a ratio of 1:9 (v/v), and the obtained solution was diluted again with PBS in a ratio of 1:15 (v/v). The obtained erytrocitar solution was added in volumes of 1 mL in each centrifuge tube. The chitosan samples were previously dissolved in 1% acetic acid and in 1% lactic acid and the measurements were realized in triplicate. The prepared samples were kept at 37°C for 12 h, then were centrifuged for 10 min at 4000 rpm and the clearsupernatant solutions were measured using a Jasco V-630 UV-Visspectrophotometer at λ = 540 nm (Zhou et al., 2017).

RESULTS AND DISCUSSIONS

The physical-chemical characteristics of the extracted chitosan sample were performed in duplicate and are presented as follows: deacetylation degree of 94.1 ± 3.5%, viscosity of 209 ± 13 mL/g and ash content of 0.29 ± 0.1%. Consequently, the obtained sample presents a high deacetylation degree and a medium molar mass, and the results are comparable with those obtained by Kucukgulmez et al. (2011) in their study. The high deacetylation degree gives a more reactive character to chitosan in acidic medium, thus contributing to an increase of the antioxidant activity (Rasti et al., 2017). Furthermore, the deacetylation degree values have a significant influence on the biological activity of the extracted chitosan and on its solubility in various acidic solutions (Soares et al., 2009). The viscosity gives informations about the average molar mass and the aggregation capacity of the biopolymer and it strongly depends on the solvent used for solubilization (Younes and Rinaudo, 2015). Generally, the ash content gives information about the total amount of inorganic material, thus, the low ash content obtained for the extracted chitosan could be suitable for biomedical applications (F2103–01, 2006).

Therefore, the obtained properties of chitosan led to the hypothesis for testing its antioxidant and hemolytic activities.

Acetic acid is the most used solvent for chitosan solubilization but also the lactic acid, which is widely used in biological applications (Younes and Rinaudo, 2015). Therefore, both solvents were compared for the antioxidant activity evaluation of chitosan.

Two methods for the antioxidant activity evaluation of chitosan were used. First, the antioxidant activity was determined by ABTS method which consisted in subjecting the chitosan samples to long-life radicals generated by the reaction of ABTS with an oxidizing species represented by the potassium persulfate, then the solution was normalized to 0.7 ± 0.1 absorbance at 734 nm. The absorbance of the chitosan solutions was measured at 734 nm and the results showed that chitosan dissolved in 1% acetic acid presented a higher inhibition percent than chitosan dissolved in 1% lactic acid, for chitosan concentrations ranging between 250 and 2000 µg/mL. Though, both solvents presented similar values for the determination coefficients of R^2 = 0.99 (Figure 2).

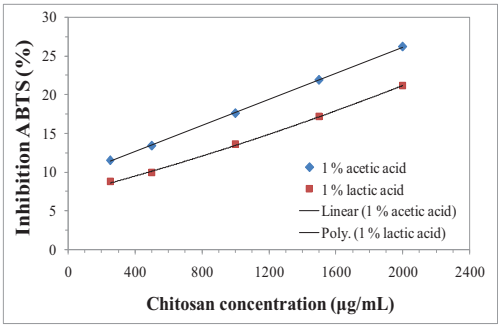

Figure 2. ABTS method evaluated for chitosan dissolved in acetic acid and lactic acid

Chemiluminiscence detection is an extremely sensitive and selective method (Klampfl, 2005), compared to other methods. The

chitosan samples were evaluated in terms of the ability to scavenge the free radicals of short life generated by the reaction of luminol with hydrogenperoxide in the presence of a catalyst to yield 3-aminophthalate in an excited electronic state, which is a light-producing emitter. By hydrogen atom donation the antioxidant samples can quench the activity of the hydrogen peroxide and, thus, lead to the inhibition of the hydrogen peroxide-induced chemiluminiscence (Klampfl, 2005; Zhong and Shahidi, 2015).

The results showed that chitosan dissolved in 1% acetic acid presented a higher inhibition percent than chitosan dissolved in 1% lactic acid, for the same chitosan concentrations used at chitosan concentrations ranging between 50 µg/m Land 400 µg/mL. The equation obtained for acetic acid had a determination coefficient of R^2 = 0.98, while for the lactic acid it was obtained R^2 = 0.99, which reveals a better correlation of the experimental data (Figure 3). Usually, the increse of chitosan concentration leads to an increase of the antioxidant activiy (Rasti et al., 2017; Abdel-Ghany and Salem, 2019), but also the antioxidant activity is strongly influenced by the solvent used for solubilization (Charernsriwilaiwat et al., 2012).

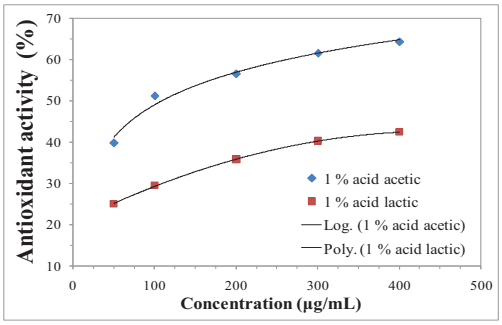

Figure 3. Chemiluminiscence method evaluated for chitosan dissolved in acetic acid andlactic acid

The preliminary results showed that chitosan captures the long and also the short term life radicals and, based on these results, it could be used in aquaculture as feed additive in fish diets due to its capacity of improving health by reducing or preventing the oxidation processes (Abdel-Ghany and Salem, 2019).

Hemolysis represents an important test for biomaterials evaluation and it is calculated as lysis percent of red blood cells (RBCs) produced as a result of the interaction with the biomaterial. The hemolytic activity for chitosan dissolved in 1% acetic acid (AC) solvent showed a more intense activity compared with 1% lactic acid (AL) solvent. For the same chitosan concentration it can be noticed that acetic acid produced a 2-3 times higher hemolytic activity than the lactic acid. The obtained results are presented in Figure 4.

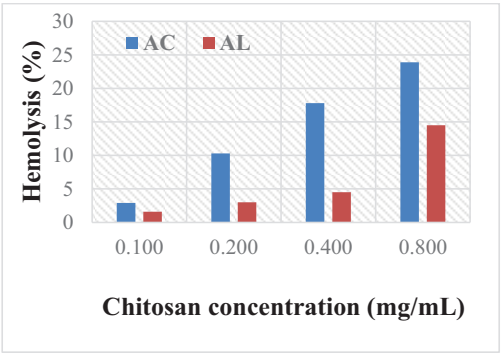

Figure 4. The hemolyticactivity (%) evaluated for the extracted chitosan sample

The activity values below 5% (F756–00, 2000) are classified as slightly hemolytic for chitosan dissolved in lactic acid at concentrations below or equal than 0.4 mg/mL, but also for the chitosan samples dissolved in acetic acid at 0.1 mg/mL concentration. Similar results of hemolytic activity were obtained by Zhou et al. (2017) for chitosan dissolved in solutions of acetic acid. Though, according to F756–00 standard (2000) the values that are higher than 5% classifies chitosan as having hemolytic effects, which occurs for the chitosan samples dissolved in acetic acid at concentrations higher or equal than 0.2 mg/mL.

Therefore, the obtained results highlighted a potential use of chitosan in biomedical applications at certain concentrations that do not affect the integrity of the red blood cells. Also, the solvent used for chitosan sample preparation is an important factor that needs to be chosen deppending on the applications and the expected results.

Though, the hemolytic effect may be lowered if chitosan could be combined with materials such as polyethylene that have no interaction or low interaction with the red blood cells (F756–00, 2000).

CONCLUSIONS

The marine waste leads to environmental problems because of its high pollution capacity. Therefore, by using the traditional chemical procedure that involves low financial resources the shrimp waste was successfully converted into chitosan.

The antioxidant capacity was evaluated using ABTS and chemiluminiscence methods, and the obtained chitosan presented the highest antioxidant activities for the samples dissolved in acetic acid for both methods used.

Hemolytic activity evaluation showed that chitosan is recommended to be used in situations that involves direct contact with blood, but only in lactic acid and at low concentrations.

The preliminary studies showed that potential applications could be envisaged by using chitosan in aquaculture as food additive, in agriculture as fertilizer or in medical field as bandage for potential infections.

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