CHARACTERIZATION AND SOLUBILIZATION OF CHITOSAN FROM THE ORIENTAL HORNET (VESPA ORIENTALIS)

By
MAGDA H. RADY¹, EMAN E. ESSA¹, SHIREEN A. M. MA’MOUN¹, SHIMAA A. A. MO’MEN¹, MOHAMMAD S. SALAMA¹, EMAD M. S. BARAKAT¹, AND WAEL S. I. ABOU-ELMAGD²

Department of Entomology¹ and Department of Chemistry², Faculty of Science, Ain Shams University, Cairo 11566, Egypt

(*Correspondence: eman_smile2008@yahoo.com)

Abstract

Chitosan was extracted from the oriental hornet, Vespa orientalis (L.), Vespinae, Order; Hymenoptera, as a new source of insect chitosan, using chemical methods. We assessed the physical properties of the wasp’s chitosan using different approaches including: preliminary color-change identification, Fourier Transform Infrared spectroscopy (FTIR), X-ray diffraction and Nuclear Magnetic Resonance spectroscopy (¹H-NMR). The results showed that it gave a higher purity, solubility and Degree of Deacetylation (DD) compared to crustacean chitosan. Also, it is soluble in a very low concentration of acetic acid from 0.25% to 0.5% while that of Crustacea was soluble in more than 1% acetic acid, so the oriental hornet could be a novel alternative source of higher pure and more soluble chitosan.

Keywords: Egypt, Oriental hornet, Chitosan solubility, Deacetylation degree.

Introduction

Chitosan is one of the most frequently cited polymers in the scientific research dealing with a wide range of biopharmaceutical and biomedical applications together with food science and technology (AbdElhady et al, 2012; Bellich et al, 2016). Chitosan is derived by the deacetylation of chitin (QI et al, 2004). Chitin is one of the major components of the cuticle, trachea, and peritrophic matrix of insects (Nemtsev et al, 2004; El-Shehaby et al, 2011).

The majority of studies of chitin and chitosan refer to the isolation and properties of these substances from the shells of commercially harvested crustaceans, because this is the most readily available material for large-scale manufacturing. However, recent works describe the obtaining of chitin and chitosan from the cuticle of insects. At first glance, these sources are not appropriate for industrial manufacturing; however, certain species of insects can be used for accumulation of a large amount of chitin-containing material that is suitable for industrial processing. These are insects that can be reared or obtained through field collection in accumulation as wasps, honeybees, silkworms, and synanthropic flies (Nemtsev et al, 2004). This new source provide no seasonal limitation accessibility to raw materials, low inorganic salt content and no regional limit to industrial production. Due to the availability of the corpses of wasps in the Apiculture fields in a huge amount manufacturing can be done on a large scale (Ibitoye et al, 2018).

In previous research on chitosan, the most common solvents for chitosan dissolution have been acetic acid solution (Chen et al, 2007). The difficulties in solubilizing chitosan is the main problem facing many researchers even in acetic acid solution, this problem hinder many applications including preparation and synthesis of chitosan nanoparticle compounds so a new method will be discussed in the current work to improve chitosan solubility.

In the present study, Chitosan was extracted from the oriental hornet (Vespa orientalis, a social insect of the family Vespidae), which is a primary pest to honey bees, attacking bee colonies to obtain honey and animal proteins (Glaim, 2009). The oriental hornet physical properties were characterized by using a variety of approaches includ-
ing: preliminary color-change identification, FTIR, X-ray diffraction and $^1$H-NMR. The improving its solubility was studied (Castro et al., 2012; Murugan et al., 2015; 2017).

Materials and Methods

The local resource used to obtain chitin was Vespa orientalis collected from the Apiculture Research Department, Plant Protection Research Institute, Agricultural Research Center, Dokki, Egypt. Wasps were collected after sting removal. Chitosan 85% degree of deacetylation and 100 cps viscosity was purchased from Shanghai Bioscience & Technology Co. Ltd., China. All the chemicals used (for example: iodine, potassium iodide, sulfuric acid, ethanol, hydrochloric acid, sodium hydroxide etc.) were purchased from Sigma-Aldrich.

The chitin was extracted from Vespa orientalis following the standard procedure mentioned in (Majtán et al., 2007) with little modifications. Wasps corpses were washed several times with water and dried at 50 °C overnight in a dry heat incubator, specimens were mechanically grinded in a mixer. De-mineralization step was carried out by treatment with 1.0 M HCl solution to solid ratio 15mL/g. at 100°C for 20min. The resulted solid fraction was washed with distilled water till neutral pH value was recorded. De-proteinization was performed using alkaline treatment with 1.0 M sodium hydroxide at 85°C. Treatment was repeated several times during 24 h and washed with distilled water until pH value became neutral. Pigment traces responsible for the brown color of this product were removed using a mild oxidizing treatment (H$_2$O$_2$/33% HCl 9:1, v:v) (Arbia et al., 2013). Finally, lightly brown chitin was washed with distilled water and dried at 50°C in a dry heat oven. The resulted chitin was then washed with distilled water until neutralization.

The extracted chitin from Vespa orientalis was treated with 50% NaOH at 100 °C for 2 h on a hot plate. The mixture was stirred after some times for homogenous reaction. Samples were then cooled for 30 min at room temperature, after cooling samples were washed continuously with 50% of NaOH and filtered in order to retain the solid matter. This solid matter was further washed with deionized water and dried in oven at 120 °C for 24 hrs (Prakasam and Azariah, 1975). The modification in this step (de-acetylation) was achieved by the repetition of deacetylation process for additional time only for the obtained poorly soluble chitosan in 1% acetic acid, this time differ according to solubility degree of the produced chitosan (Majtán et al., 2007).

Process of deacetylation of chitin to chitosan was ensured using confirmatory test (Kumar and Verma, 2012), 5 ml of Iodine solution (I$_2$)/potassium iodide (KI) solution was added to a test tube with traces of chitosan and another one without chitosan, considered as control, the solution takes the yellow color of iodine. Concentrated sulphuric acid (H$_2$SO$_4$) was added to each tube; the change of color from yellow/brown to dark purple confirms the presence of chitosan.

The wasp chitosan powder (0.1g in triplicate) were placed into a centrifuge tube (known weight) then dissolved with 10ml of 2, 1, 0.5, 0.25 % acetic acid by hand shaking for 5min. The solution was then immersed in a boiling water bath for 10 minutes, cooled to room temperature (25°C) and centrifuged at 10,000 rpm for 10 min. The supernatant was decanted. The undissolved particles were washed in distilled water (25ml) then centrifuged a 10,000 rpm. The supernatant was removed and undissolved pellets dried at 60°C for 24hr, and then weighed the particles and determined the solubility% (Sun et al, 2016).

The degree of deacetylation (DD) of chitosan was measured by titration method (Abdou et al., 2008). Chitosan (0.5 g) was weighed and dissolved individually in 20 mL 0.3 N HCL at room temperature. Distilled water (400mL) was added, and then the wasp chitosan solution was titrated with 1 N NaOH solution. A titration curve of pH ascending values vs. NaOH titration volume.
was produced. The curve's inflection points were found for each indicated transition, then, NaOH volume at each inflection point was applied to the equation (Eq. 1): DD% = 16.1(y-x)/WEq. Where W= chitosan weight used (0.5 g), X= the first inflection point on the graph of measured pH vs. titration volume, and y= the second inflection point.

FTIR spectra of chitosan from wasp were recorded with FTIR (4100Jasco-Japan) spectrophotometer. The spectral region between 4000 and 400 cm⁻¹ was scanned. Specimens were prepared as KBr pellets. Dried wasp’s chitosan powder was mixed thoroughly with KBr and then pressed to form an ultimate thin homogenous disc with a thickness of 0.5 mm. The wasp’s chitosan concentration in the samples was 2% calculated with respect to KBr.

XRD analysis estimated the crystallinity of the prepared chitosan. The XRD measurements on powder samples were done using a PANalyticalX'Pert PRO X-ray machine in Faculty of Science Ain-Shams University. X-ray source was Cu Ka radiation (45 kV, 30mA). Wasp’s chitosan samples were scanned from 26= 5-40° at a scanning rate of 4min⁻¹ and temperature 25°C (Islam et al, 2011). Intensities of the peaks at 110 lattices (I₁₁₀, at 26 = 20° equivalent to maximum.

1H-NMR spectra were measured on Varian Gemini 300 MHz spectrometer, with chemical shift (δ) expressed in ppm downfield with tetramethylsilane (TMS) as internal standard, in DMSO-d₆ and coupling constants J in Hz were measured at Microanalytical center, Cairo University.

**Results**

The results are shown in table (1) and figures (1, 2, 3, 4 & 5).

<table>
<thead>
<tr>
<th>Items</th>
<th>Before Re-deacetylation</th>
<th>After Re-deacetylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid conc.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5%</td>
<td>1%</td>
<td>2%</td>
</tr>
<tr>
<td>Wasp chitosan solubility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20%</td>
<td>43.1%</td>
<td>69.9%</td>
</tr>
<tr>
<td>Commercial chitosan solubility</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22%</td>
<td>75.7%</td>
<td>98%</td>
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<td>98%</td>
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Solubility degree of wasp chitosan was measured using different concentrations of acetic acid in atrial to increase chitosan solubility wasp chitosan after extraction process demonstrated weak solubility at concentrations (0.5,1,2%)of acetic acid while after an additional step of deacetylation, the solubility increased to 100% at (0.25-2%) acetic acid. This excellent solubility was done after repeating the deacetylation step, the co-mmercial chitosan (without any treatment), showed slightly moderate solubility 75.7% at 1% acetic acid and 98% at 2% acetic acid.

**Discussion**

The recovery rate of chitosan extracted from the oriental hornet was 12.2%; whereas that from the black tiger shrimp head was 14.6% (Tsai et al, 2011), however yield was moderately low as compared to crabs and other crustaceans, This may be due to less amount of chitin in exoskeleton of the oriental hornet. By comparing the reported insect species the oriental hornet gave much higher yield comparing to Periplaneta americana which gave 2.0% and 26.2% for the blowfly, Chrysomyia albiceps (Song et al, 2013). The change of the pale yellow color of the extracted chitosan (Fig. 1a) and the whole solution was changed to the deep purple color by adding the concentrated sulphuric acid that proved the success of chitosan extraction (Fig.1b).

One step of deacetylation was sufficient to convert chitin into chitosan in all tested samples but the rate of solubility was less than that of crustaceans but after increasing time of deacetylation as an additional step of deacetylation the solubility was enhanced. The difficulties in solubilizing chitosan is the main problem facing many researchers as it hinder many applications including preparation and synthesis of chitosan nanoparticle compounds.

The solubility of chitosan in acetic acid is a mark of its purity. The lower acid concen-
tration used, the higher the purity of chitosan (Mohammed et al., 2013). The purity level of chitosan was a factor which affects not only the biological properties like immunogenicity or biodegradability, but also has a profound effect on its solubility and stability (Szymańska and Winnicka, 2015). Most crustacean chitosan and that isolated from different sources dissolved in 1-3% acetic acid (Ghaly et al., 2018). While the isolated wasp chitosan completely dissolved in 0.25-0.5% acetic acid and this is a mark that our isolated wasp chitosan has a higher purity than the commercial one, this excellent solubility which wasn’t obtained from any other chitosan due to the entered modification in the isolation procedure (repeating deacetylation step for another period after complete isolation).

The degree of deacetylation (DD) of chitosan is the most important parameter that influences their various properties including biological, physicochemical and mechanical properties and it depends on the method of isolation and the reaction conditions should be taken into consideration prior to the use of chitosan as drug delivery system (Khan et al., 2002). No doubt, the DD of wasp chitosan exhibited the highest (92-93%), while commercial chitosan had lower DD (83.8-85.8%).

In the present study, FTIR spectra of chitosan obtained from wasp and crustacean chitosan were recorded with a Nicolet FT-IR (4100Jasco-Japan). The chemical structure of the chitosan derived from Vespa orientalis was confirmed by FTIR analysis. FTIR Spectrum of chitosan showed characteristic peaks at 3500, 3300 and (br) 3448 Cm⁻¹ which indicated symmetric stretching vibration of OH and amine groups. Absorption peaks at 2918 and 2884Cm⁻¹ indicated the presence of CH stretch, Absorption band at 1654 was due to C=O stretching (amide I) and peaks at 1081, 1033 corresponding to C-O stretching. Peak at 894.91 was a ring stretching characteristic for β-1-4 glycosidic linkage. FTIR spectra of wasp chitosan was to a large extent similar to the FTIR Spectra analysis of chitosan carried out by many authors (Brugnerotto et al., 2010; Anicuta et al., 2010; Ramya et al., 2012; Marei et al., 2016).

In the present study, XRD patterns of the chitosan from shrimp exhibited two sharp (9.4 & 20.2°) and two faint (22.0 & 26.8°) diffraction peaks. While that from wasp showed two sharp peaks at 10.4, 20° and one faint 22.1°. Similar peaks were reported in a chitin and chitosan structures obtained from different organisms such as insects, crustaceans, entozoa and fungi (Ifuku et al, 2011; Wang et al, 2013; Kaya et al, 2014). The two sharp peaks appeared at around 10 and 20° that corresponding to the (0 2 0) and (1 1 0) planes of the crystalline lattice are classically characterized chitosan (Lai et al, 2010; AbdElhady, 2012; Marei et al, 2016).

In the present study, ¹H-NMR spectrum showed singlet peaks at 1.8 and at 4.6 ppm corresponding to CH₃ of acetyl group and H-1(Ac) of acetylated chitosan respectively, beside the characteristic peaks of deacetylated chitosan at 2.9, 3.4-3.9 and 4.7 ppm.

Undoubtedly, more support for the structure and the ratio of acetylated and deacetylated chitosan were gained from ¹H-NMR revealed that the percentage of methyl group of the acetamido group of acetylated chitosan at δ=1.8 ppm decreased and degree of deacetylation increased with increasing time of deacetylation (Vongchan et al, 2002).

**Conclusion**

The characteristics of produced wasp chitosan were in accordance with the commercial standard. The investigation revealed that the wasp has lower yield of chitin of 12.2% but of higher purity compared to that of the shrimp. The degree of deacetylation (DDA) was determined. It was found that the DD of chitosans derived from wasp cuticles are found to be comparable (92-93%) and higher than that of chitosan from shrimps (75%). X-ray powder diffraction (XRD) showed that the wasp’s chitosan had higher crystallinity than that of the shrimp.
Chitosan with such properties has several commercial applications and greater scope of industrial applications and these results encourage us to continue the study of this kind of chitosan. Further studies in this area are ongoing progress and will be published in due time elsewhere.

Author Contribution: Magda Rady, Wael Abou-Elmagd and Eman Essa conceived the idea and designed the plain. Eman Essa, Shreen Ma’moun and Shimaa Mo’men conducted the practical work. Mohamed Salama, Emad Barakat and Shreen Ma’moun contributed material. Eman Essa, Magda Rady and Wael Abou-Elmagd wrote the manuscript. All authors approved the manuscript.

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ORCID:
Essa, EE, http://orcid.org/0000-0003-1230-6333

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Explanation of figures

Fig. 1: a- Pale yellow powder of experimentally obtained chitosan. b- Bright and deep purple color appeared on addition of iodide/potassium iodide aqueous solution (0.03% w/w) and concentrated sulfuric acid aqueous solution (1%, w/w) to chitosan sample. Fig. 2: a- FTIR spectra of chitosan from oriental hornet. b- Crustacean chitosan from traditional sources. c- Overlay between two spectra. Fig. 3: XRD patterns of chitosan from a- shrimp (Ramya&Mahalakshmi 2012) and b- Oriental wasp. Fig. 4: 1H-NMR spectra of the chitosan prepared from the oriental hornet. (A)after 2 hr deacetylation (B) after re-deacetylation (4 hr). Fig. 5: Deacetylated and acetylated monomers of chitosan.