Extraction and characterization of high purity chitosan by rapid and simple techniques from mud crabs taken from Abbottabad

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Abstract: Chitosan and chitosan based materials offer diverse applications in the field of biotechnology, nanotechnology, pharmaceuticals, environmental protection and tissue engineering due to their various biological and physicochemical properties. Major sources of chitosan are shrimps, crabs and lobsters. Properties of chitosan differ with the degree of deacetylation and the molecular weight. Researchers are investigating to produce high quality chitosan in cost effective and time efficient way which was the aim of present study. The exoskeleton of mud crabs, taken from Abbottabad, was demineralized with 2mol/dm^3 H_2SO_4 solution for 4hour and then, deproteinized with 2mol/dm^3 NaOH solution for 4hour at room temperature. Yield of crude chitin was 78% which was deacetylated with 55% NaOH solution at 110^{o}C for 4hour to obtain chitosan. After precipitation, the yield of pure chitosan form the crab shell was 39%. The degree of deacetylation of chitosan was 92% measured by potentiometric titration and the molecular weight was 1.2×10^6g/mol (1200KD), determined by viscometric method. We concluded that a high quality chitosan can be produced at commercial level in Pakistan by rapid and simple techniques.

Keywords: Chitin, chitosan, degree of deacetylation, molecular weight, potentiometeric titration.

INTRODUCTION

Chitin is cellulose like naturally occurring polysaccharide containing N-acetyl glucosamine units (Kumar and Majeti, 2000; Sakthivel *et al*., 2015). Deacetylation of chitin produces chitosan, a copolymer of N-acetyl glucosamine and D-glucosamine units, which is white, inelastic, nitrogenous polysaccharide (Divya *et al*., 2014; Younes I. and Rinaudo M., 2015). Chitosan and chitosan based materials like nanoparticles, hydrogels, films, microspheres and fibers offer diverse applications in the field of biotechnology, nanotechnology, pharmaceuticals, tissue engineering, genetic engineering, environmental pollution protection, paper, cosmetics, dyes and food industries, biomedical products, photography etc. due to their various biological properties like biodegradability, biocompatibility in both plant and animal tissue, non-toxicity, antimicrobial and anti-inflammatory activity, and physicochemical properties like polycationic electrolyte, solubility, adsorption and coagulation (Cheung *et al.*, 2014; Divya *et al*., 2014; De Alvarenga, 2011; Thakur V. K. and Thakur M. K., 2014; Vakili M. *et al.*, 2014). Chitosan can be transformed into gels, beads, colloids, films and capsule (Sakthivel *et al*., 2015). Properties of chitosan like solubility, reactivity and biodegradablility etc. depends on degree of deacetylation of chitosan (protonated amino group in the polymer chain) and molecular weight (Yuan *et al*., 2011). Chitosan is insoluble in water, organic solvents and bases. It is soluble in acidic medium below pH of 6.2 (De Alvarenga, 2011).

Major sources of chitin and chitosan are crustaceans, arthropods, mollusks, fungi and mushrooms (Arbia *et al*., 2013; Kaya *et al.*, 2014; Sakthivel *et al*., 2015). The amount of chitin varies from organism to organism e.g. chitin obtained from the crustacean crabs is 60-70% (Arbia *et al*., 2013). The crustaceans, like crabs, shrimps and lobsters are the commercial sources of chitosan production due to its high content (Sakthivel *et al*., 2015; Das *et al*., 2010). Commercial preparation of chitin from the exoskeleton is usually performed by demineralization with acid treatment like HCl, H_2SO_4, HNO_3 and CH_3COOH and deproteinization with alkali treatment. Chitin is converted into chitosan by deacetylation with alkali treatment at high temperature (Das *et al*., 2010; Kaur S. and Dhillon G. S., 2014). Increased processing time and temperature during deacetylation process reduces the molecular weight and increases amine groups i.e. DD% (Yuan *et al*., 2011; Maji *et al*., 2013). Properties of chitosan differ with the molecular weight and the degree of deacetylation. High molecular weight chitosan creates more viscous solution which is less favored. On the other hand by increasing DD% the molecular weight decreases and softness increases which is more favored (Divya *et al*., 2014; Wang *et al*., 1991). It was also observed that high DD% chitosan contains smaller amount of ash and protein contents (Yuan *et al*., 2011). Therefore, chitosan can be purified by precipitation method to obtain high DD% (Wang *et al*., 1991).

Degree of deacetylation can be measured by using different titration techniques, IR spectroscopy, Gas chromatography, GPC, UV spectrophotometry, H^1 NMR and C^{13} NMR etc. depending upon the facilities, time, and cost. DD varies with the deacetylation process and the measurement technique (Wang *et al*., 1991). Studies reported that titration method gives same results as IR

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spectroscopy and NMR, which is convenient and cost effective (Wang *et al*., 1991). Different methods can be used to determine molecular weight, like intrinsic viscosity measurement, Gel Permeation Chromatography (GPC), Static Light Scattering (SLC) and HPLC etc. (Knaul *et al*., 1998; Kasaai *et al*., 2000). The present attempt was made to extract high quality chitosan from mud crabs by rapid and simple techniques, within limited laboratory facilities and instrumentation.

MATERIALS AND METHODS

Sample collection and preparation

This study was conducted in Laboratory of Chemistry Department, Government Post Graduate College No.1 Abbottabad during April 2016. Mud crabs were collected from fresh water canals near Thandiani, Abbottabad and the approval for study was taken from marine department of Pakistan. Exoskeleton was separated, washed, shade dried, and crushed into powder.

Demineralization and deproteinization

Demineralization was carried out by dissolving 10 g of dried crab shell powder in 300ml of 2mol/dm^3 H_2SO_4 solution with a ratio of 1:30, at room temperature. The solution was kept for 4 hour. Acid was drained off and the sample was washed with distilled water until neutral pH was reached. After demineralization the sample was dissolved in 300ml of 2mol/dm^3 NaOH solution (1:30), at room temperature, for deproteinization. The sample was again kept for 4hour and washed until neutral pH was reached (Paul *et al*., 2014; Burrows *et al*., 2007).

Deaectylation

The obtained chitin was dissolved in 55% NaOH solution, with a ratio of 1:30 and boiled at 110°C for 4hour. After cooling the sample was washed with 40% NaOH and filtered to obtain chitosan (Paul *et al*., 2014; Burrows *et al*., 2007).

Precipitation

Chitosan was purified by precipitation method. The chitosan obtained was dissolved in 2% acetic acid solution with 1:100, for 4hour with constant stirring. The solution was obtained in a separate flask and 0.5mol/dm^3 NaOH was added. The solution was kept for 5-10minutes so that precipitation occurs. The precipitates were filtered, washed and dried at 110°C for 1 hour (Maji *et al*., 2013).

Measurement of DD by potentiometric titration

Chitosan of 0.2g was dissolved in 20ml of 0.1mol/dm^3 HCl solution and 50ml of distill water was added. After continuous stirring, when the chitosan was completely dissolved, the solution was titrated against 0.1mol/dm^3 NaOH solution. The pH of chitosan solution was measured after each addition of standard NaOH solution. The two inflexion points: V_1 corresponding to neutralization of HCl, and the V_2 corresponding to neutralization of the ammonium ions from chitosan were determined by the graph obtained by plotting pH versus volume of NaOH solution. The degree of deacetylation was calculated by using equation 1 (Biskup *et al*., 2012).

$$DD[\%] = 2.03(v_2\text{-}v_1)/m+0.0042(v_2\text{-}v_1) \quad (1)$$

Where, m is the weight of sample, 2.03 is the coefficient resulting from the molecular weight of chitin monomer unit and 0.0042 is the coefficient resulting from the difference between molecular weights of chitin and chitosan monomer units (Biskup *et al*., 2012).

Molecular weight determination by Ostwald viscometric method

For the determination of molecular weight, five different concentrations (0.2-0.6%) of chitosan solution were prepared in 0.1mol/dm^3 CH_3COOH and 0.2mol/dm^3 NaCl solvent. The time of flow for solvent and solution of each concentration was measured by using Ostwald viscometer at room temperature. The data was used to measure the relative viscosity, reduced viscosity and inherent viscosity. By applying equation 2, inherent viscosity was measured.

$$\eta_{inh} = \ln\eta_{red}/c \quad (2)$$

The inherent viscosity was plotted against chitosan concentration and the value of intrinsic viscosity (η_{int}) was calculated by extrapolating graph of inherent viscosity to zero. The molecular weight (M) was calculated by applying Mark-Houwink-Sakurada (MHS) equation (3), where value of k=1.81×10^{-3}ml/g and a=0.93 depending upon type of solvent and temperature (Maji *et al*., 2013). The molecular weight was calculated by applying Mark-Houwink-Sakurada (MHS) equation. (Maji *et al*, 2013; Kasaai *et al*., 2000).

$$\eta_{int} = kM^a = 1.81 \times 10^{-3} M^{0.93} \quad (3)$$

RESULTS

Chitosan extraction from crab exoskeleton

Mud crabs were collected from fresh water canals near Thandiani, Abbottabad. The 10g dried powder of crab exoskeleton was demineralized with 2mol/dm^3 H_2SO_4 solution (1:30) for 4hours and then deproteinized with 2 mol/dm^3 NaOH solution (1:30) for 4hours at room temperature. Crude chitin of 7.8g (78%) from crab shell was obtained, represented in fig. 1. Deacetylation of chitin was carried out at 110°C for 4 hour with 55% NaOH solution. Chitosan was purified by precipitation method to remove remaining impurities. Dried and pure chitosan of 3.9g was obtained, shown in fig. 2. The chitosan yield was 39% from the crab shell and 50% as compared to crude chitin. High chitosan yield (39%) in the present study may be due to the high chitin yield (78%).

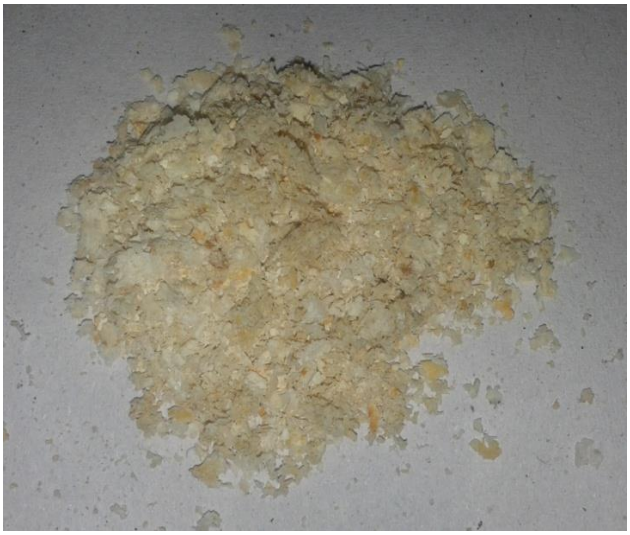

Fig. 1: Chitin produced from exoskeleton of mud crabs

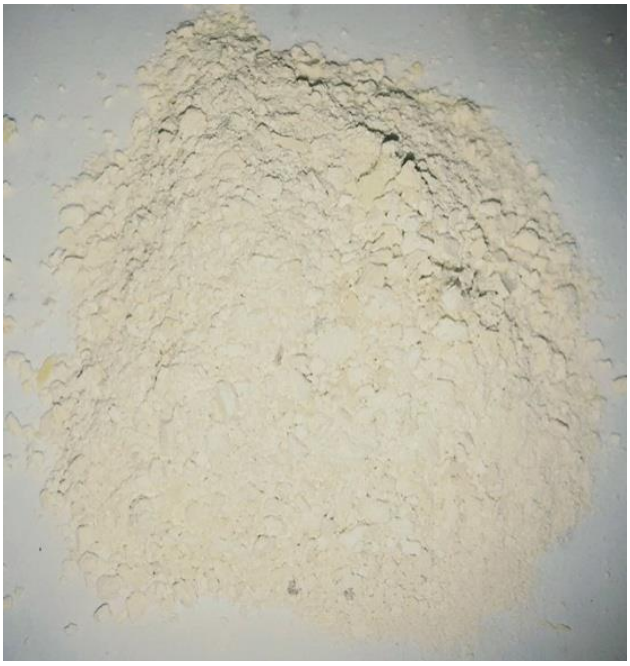

Fig. 2: Chitosan produced after precipitation

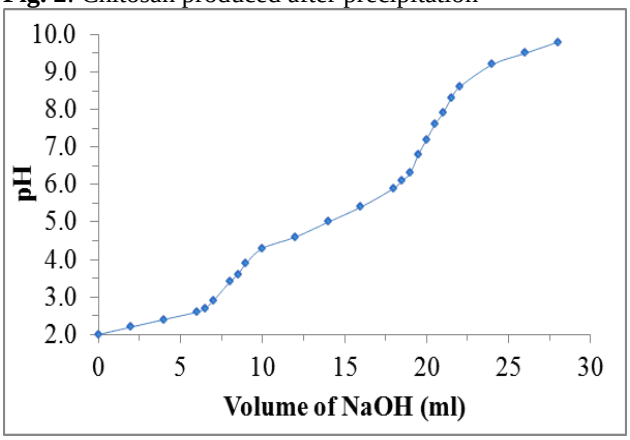

Fig. 3: Plot of pH of chitosan solution versus volume of NaOH solution

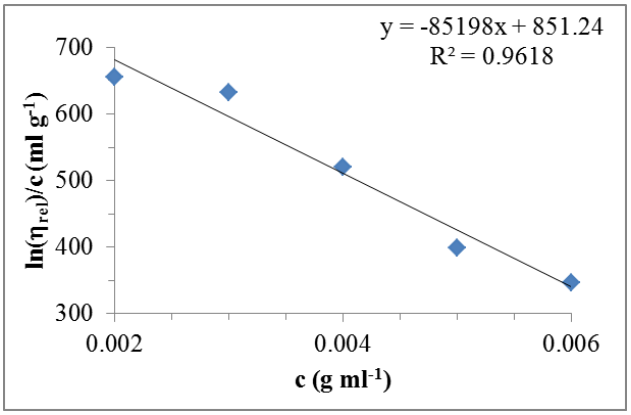

Fig. 4: plot of inherent viscosity of chitosan versus concentration

Degree of deacetylation of chitosan

Degree of deacetylation was calculated by potentometric method. Studies reported that titration method gives same results as IR spectroscopy and NMR, which is convenient and cost effective (Wang *et al*., 1991).The two inflexion points: V_1=8.8ml and V_2=20ml, were determined by the graph obtained by plotting the pH versus volume of NaOH solution, represented in fig. 3. The degree of deacetylation was calculated by using equation 1. The DD% of produced chitosan was 92%. The DD of chitosan varies within a range of 30-100% depending upon deacetylation and characterization techniques.

Molecular weight of chitosan

Molecular weight of chitosan was determined by viscometric method. The inherent viscosity was plotted against chitosan concentration and the value of intrinsic viscosity (η_{int}) was calculated by extrapolating graph of inherent viscosity to zero as shown in fig. 4. By applying Mark-Houwink-Sakurada (MHS) equation 3, the calculated molecular weight was 1.2×10^6g/mol, (1200 KD) for obtained chitosan of 92% DD.

DISCUSSION

During demineralization process it was observed that CO_2 was ceased to evolve after 4hour which is the indication of removal of $CaCO_3$. During deproteinization maximum discoloration was observed within 4hour. Past studies reported that yield of chitin from crab's shell is 60% to 70% (Arbia *et al*., 2013). The present high yield (78%) may be due to protein contents or impurities left in the chitin due to incomplete deproteinization. The remaining proteins, if any, were removed during deaectylation process i.e. the reaction of NaOH with residual proteins (Wang *et al*., 1991). The purified chitosan from chitin was 50% and as compared to crab shell it was 39%. About 50-60% yield of chitosan from the chitin was also reported by Wang *et al*. (1991). The lower limit of yield (50%) from chitin may be due to the protein contents and

impurities that were removed during deacetylation and precipitation process. But, as a whole, the yield of chitosan was very good i.e. 39%. The reported yield of chitosan from whole crab shell is 30-36.7% (Divya *et al*., 2014). High chitosan yield (39%) in the present study may be due to the high chitin yield (78%), as it varies between different crabs according to species and seasons (Sakthivel *et al*., 2015).

In our experiment 92% DD was achieved. The DD of 87% was reported by Paul *et al*., 2014. According to Wang *et al.* (1991) DD increases with reaction time and temperature during deacetylation process. Yuan *et al.* (2011) reported that high DD chitosan contains lesser amount of ash and protein contents. Therefore, the present data represents high quality and purity of chitosan. Molecular weight of the extracted chitosan was 1.2×10^6g/mol (1200 KD). By applying same solvent, temperature and constant values, Roberts *et al.* (1982) reported molecular weight of 6.3×10^5g/mol for 80% DD and Maji *et al.* reported 8.78×10^5g/mol for 84% DD (Kasaai *et al*., 2000). Deviation of our results from the given data may be due to the variation in degree of deacetylation as the values of constant 'k' and 'a' also depends on DD% along with solvent and temperature (Kasaai *et al*., 2000; Knaul *et al*., 1998). The molecular weight reported by Wange *et al.* (1991) was 1.2×10^6 g/mol for 91% DD, which is almost similar as we observed for 92% DD extracted chitosan.

CONCLUSION

The properties of chitosan vary depending upon natural origin, source and extraction process. In the present study the extraction was carried out by demineralization, deproteinization and deacetylation and purification by precipitation method by using chemicals easily available in laboratory. The extraction was carried out in a very shorter time period in high yield of 39% than that reported in literature. The high purity of chitosan with 92% DD was obtained by modifying the previous methodologies to make this process time efficient. The Chitosan was characterized by simple laboratory techniques and found in accordance with the commercial standard. The degree of deacetylation of extracted chitosan was determined by titration method. The molecular mass of chitosan was determined by viscosity method and was found to be 1200KD. Therefore, keeping in view the diverse applications of chitosan, it can be industrialized with minimum resources in Pakistan.

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