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Extraction of chitin from chitosan from exoskeleton of shrimp for application in the pharmaceutical industry

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ABSTRACT

Chitosan is an amino polysaccharide prepared by processing shrimp waste (shell) which involves partial deacetylation of chitin. Chitosan, a versatile natural polysaccharide, the second most abundant natural polymer. Many biochemists have found that chitosan as biocompatible, biodegradable and non toxic which made wide applicability in conventional pharmaceuticals as a potential formulation excipient. In this research mainly the focused on the synthesis of chitosan which is suitable for the pharmaceutical industry especially in the designing delayed and controlled drug delivery systems. The main study focused on preparation low molecular weight of the chitosan suitable for pharmaceutical industry. The crude chitin was collected from exoskeleton of *Triopslongicaudatus* and *Triopscancriformis* specimens which are then processed to obtain chitosan. The chitosan yield was found to be 35.49% and it was analyzed for its physiochemical parameters.

Key Words: Chitin, extraction, purification, characterisation, pharmaceutical grade chitosan.

INTRODUCTION

Chitosan, a natural polysaccharide, is being widely used as a pharmaceutical excipients (Singla Struszczyk, 2002). It is obtained by the partial deacetylation of chitin, natural polymer composed of randomly distributed β -(1-4)-linked D-glucosamine. It consists of two types of monomers; chitin-monomers and chitosanmonomers. Chitin is a linear polysaccharide consisting of (1-4)-linked 2-acetamido-2-deoxy-b-D-glucopyranose. Chitosan is a linear polysaccharide consisting of (1-4)-linked 2-amino-2-deoxy-b-D-glucopyranose (Tomihata and Ikada, 1997; Roberts, 1992).

The amino group in chitosan has a pKa value of ~6.5, thus, chitosan is positively charged and soluble in acidic to neutral solution with a charge density dependent on pH and the percentage of degree of

deacetylation- value. In other words, chitosan is bioadhesive and readily binds to negatively charged surfaces such as mucosal membranes. Chitosan enhance the transport of polar drugs across epithelial surfaces, and is biocompatible and biodegradable. Purified qualities of chitosans are available for biomedical application (Cho *et al.*, 1998).

Chitosan inspite of its unique properties is proving its efficacy in various dosage forms such as bioadhesive nature, hydrophilic macromolecule drug carrier, effective carrier in drug targeting to brain, transdermal films and wound healing biodegradable grafts, hyperlipidemic, antimicrobial and stabilizing constituent of liposomes. Chitosan for its potential makes it as a promising candidate as pharmaceutical excipient (Muzzrelli *et al.*, 1996; Lehr *et al.*, 1992).

The presence of a number of amino groups permit chitosan to chemically react with anionic systems, thereby resulting in alteration of physicochemical characteristics of such combinations some of which include binding, disintegrating and tablet coating

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properties (Frizakepsutlu *et al.*, 1999). The polymer in another form i.e available as d-glucosamine commercially with the slightest chemical modification has also been investigated as a potential treatment for arthritis. Chitosan alone is biologically active which is efficient in managing dietary treatments and also exhibit pharmacology as antimicrobial, hypocholesterolemic and would healing. Its unique structure makes opens its applicability as promising candidate not only as pharmaceutical aid but also in specific drug targeting and delivery. This research mainly focuses on the synthesis of pharmaceutical grade chitosan confining to predetermined quality attributes of its physiochemical properties such as gram molecular weight, viscosity, the extent of deacetylation, reminisces after burnt, water log content, present of heavymetals etc.

MATERIALS AND METHODS

Sample preparation

The shrimps were obtained from *Penaeus carinatus* and *Penaeus monodon* specimens collected from Suryalanka coast (Lat.15.83454°N Long. 80.50507°E) and the shell and operculum are removed from the animal. The shrimps exoskeletons collected are placed in Ziploc bags and refrigerated overnight. Approximately 1500 grams of crushed shrimp's exoskeletons wet samples were placed on foil paper and measured using a balance. The shrimp exoskeletons were crushed into smaller pieces using a meat tenderizer. The samples were oven-dried for 4 consecutive days at 65°C until constant weight. The dry weights of the samples were determined to be 1269 grams. The obtained shrimp is made into 4 equal parts for efficient material handling (Toan, 2009).

Extraction of chitin and chitosan

The chitin and chitosan sequence involves washing of crushed exoskeletons. crushed shrimps exoskeletons were placed in 1000 ml beakers and soaked in boiling sodium hydroxide (2 and 4% w/v) for one hour in order to dissolve the proteins and sugars thus isolating the crude chitin. 4% NaOH is used for chitin preparation, concentration used by the scientists at the Sonat Corporation (Lertsutthiwong *et al.*, 2002). After the samples are boiled in the sodium hydroxide, the beakers containing the

shrimp shell samples are removed from the hot plate, and allowed to cool for 30 minutes at room temperature (Lamarque *et al.*, 2005). The exoskeletons are then further crushed to pieces of 0.5-5.0 mm using a meat tenderizer.

Demineralization

The grounded exoskeleton is demineralized using 1% HCl with four times its quantity. The samples were allowed to soak for 24 h to remove the minerals (mainly calcium carbonate) (Trung *et al.*, 2006). The demineralized shrimp shell samples were then treated for one hour with 50 ml of a 2% NaOH solution to decompose the albumen into water soluble amino-acids. The remaining chitin is washed with deionized water, which is then drained off. The chitin was further converted into chitosan by the process of deacetylation (Huang *et al.*, 2004).

Deacetylation

The deacetylation process is carried out by adding 50% NaOH and then boiled at 100°C for 2 h on a hot plate. The samples are then placed under the hood and cooled for 30 min at room temperature. Afterwards the samples are washed continuously with the 50% NaOH and filtered in order to retain the solid matter, which is the chitosan. The samples were then left uncovered and oven dried at 110°C for 6 h. The chitosan obtained will be in a creamy-white form (Muzzarelli and Rochetti, 1985).

Purification of Chitosan

The obtained chitosan has to be purified to make it suitable for the pharmaceutical use. The purification process was designed in three steps

- 1) Removal of insolubles with filtration
- 2) Reprecipitation of chitosan with 1 N NaOH
- 3) Demetallisation of retrieved chitosan

Removal of insolubles with filtration

1 mg/ml chitosan acetic acid 1% (v/v) solution is prepared by a magnetic stirrer until an homogenous solution is obtained. The insolubles were removed by filtration through Whatman filter paper 22µm.

Reprecipitation of chitosan with 1N NaOH

Chitosan was precipitated from filtered chitosan solution by titration with 1 N NaOH until pH value of 8.5. The chitosan obtained is washed several times with distilled water by centrifuging at 8,000 to

10,000 xg. All the above steps were carried in the presence of reducing agent Dithiothreitol, (DTT) in order to provide more consistency and reproducibility between chitosan batches for biomedical applications (any other hydroxides other than NaOH are reactive which would another step in purification if such materials are used).

Demetallisation of retrieved chitosan

Reprecipitation precedes demetallisation by the addition of 1 ml of 10% w/v Aqueous solution of sodium dodecyl sulfate (SDS) and stirring for 30 min for dissolving the protein left over finally. After leaving the solution stirring at room temperature overnight, 3.3 ml of 5% w/v ethylenediaminetetraacetic acid (EDTA) was added and stirred at room temperature for 2 additional hours for precipitation of heavy metals with EDTA. The water insoluble chitosan precipitate was collected by centrifugation at 5000xg for 30 min using REMI and washed several times with distilled water by resuspending and re-centrifugation for 30 min. the residue obtained is dried in hot air oven at 60 gently to prevent physical damage in the chain structure. The obtained dried chitosan is stored in the dessicator (Qian and Glanville, 2005).

Characterization of prepared chitosan

Molecular weight

Average molecular weight of chitosan is determined by determination of its Intrinsic viscosity by Brookfield viscometer. 1 mg/ml (w/v) concentration of chitosan solution was prepared using 1% glacial acetic acid by a magnet stirrer for homogenous mixture of the polymer solution. The viscosity of the chitosan solution was analysed using spindle RV-VI. The average molecular weight was obtained from Mark-Houwink equation:

$$\eta = KM^a$$

Where $[\eta]$ is the intrinsic viscosity, M is average molecular weight of the solution respectively and K and a are the Mark-Houwink constants specific for a given polymer (Merlina A et al., 2009).

Viscosity

Viscosity of chitosan solutions will be determined at room temperature by using a Brookfield digital viscometer (Mirzadehl et al., 2002).

Degree of deacetylation

Degree of deacetylation refers to the removal of acetyl group from the chain this is determined by potentiometric titration. Chitosan homogenous solution is prepared using dil. HCl containing 0.010 mol/L which is titrated against 0.1M NaOH. The end point is detected by the inflections of the p^H values. Two inflections were mainly noted out of which first one corresponds to neutralization of HCl and second one to the neutralization of ammonium ions for chitosan chain. The difference between two points give the amount of the amino groups in the chitosan chain (degree of deacetylation) (Zhanga et al., 2011).

$$DD\% = 100 - DA\%$$

pH

The pH measurements of the chitosan solutions will be carried out using a microprocessor pH meter.

Ash value

To determine the ash value of chitosan, 2.0g of chitosan sample is placed into previously ignited, cooled, and tarred crucible. The samples are heated in a muffle furnace preheated to 650°C for 4 hr. The crucibles are allowed to cool in the furnace to less than 200°C and then placed into desiccators with a vented top. Percentage of ash value is calculated using the following. (Monarullislama Shah et al., 2011).

$$\% \text{ Ash} = \frac{(\text{weight of residue, g})}{\text{sample weight, g}} \times 100$$

Loss on drying

Loss on drying of the prepared chitosan will be determined by the gravimetric method. The water mass loss will be determined by drying the sample to constant weight and measuring the sample after and before drying. The water mass (or weight) will be the difference between the weights of the wet and oven dry samples. (Yong Hu et al., 2002).

$$\% \text{ loss on drying} = \frac{(\text{Wet weight} - \text{Dry weight})}{\text{Dry weight}} \times 100$$

FTIR studies of chitosan

The FT-IR spectrum of the standard chitosan showed nine major peaks at the ranges of 752, 893, 1022, 1421, 1552, 1643, 2865, 2921 and 3283 cm^{-1} ; whereas the chitosan sample from *penaeusmonodon*.

Table 1: Characteristics of chitosan obtained from the *panaeusmonodon*.

| Characteristics | Chitosan |
|--------------------------|---------------|
| Chitosan yield | 34% |
| Average molecular weight | 1,599,558.029 |
| Degree of deacetylation | 89.79% |
| Viscosity | 304cps |
| pH | 8.5 |
| Ash value | 0.25% |
| Loss on drying | 9.34% |
| Heavy metals | >10ppm |

RESULTS AND DISCUSSION

The synthesis of chitosan involves various chemical steps such as preparation of the chitin from the crude shells which will be the initiation of the chitosan synthesis with the removal of the proteins in the shells followed by demineralization for the removal of the carbon and other salts present in the crude form which will be preceded by the deacetylation of the chitin that would result in chitosan. The regular chitosan is obtained by following the above steps, but a polymer of pharmaceutical grade has to fall in the region of its predetermined quality aspects and Usually commercial chitins are prepared by a first step of deproteinisation followed by a second step of demineralization. The whole parameters were tabulated in the **table1** and their respective characteristics are discussed. The chitosan yield was found to be 34% after purification of the total exoskeleton taken. In these conditions the native structure of the chitin is lost, is extracted. On the otherhand, "in which the native chain and fibrous structures are intact and stabilized, is extracted when demineralization occurred in the first step.

Early studies have clearly demonstrated that specific characteristics of these products (Mw, DD) depend on the process conditions. Taking these two facts into account, the reaction conditions must be controlled when preparing chitosan. The chitosan obtained from the above process was reported to have average molecular weight of 1,599,558.029 which literally conclude that majority of the linkage was intact instead of splitting up by the influence of the strong alkali. The purification procedure included removing insoluble contaminations and adding deproteinization and demetallization agents, SDS and EDTA, respectively, in the presence of the

Table 2: Wave length of the main bands obtained for the standard chitosan and chitosan extracted from *N. crepidularia*.

| Vibration modes | Standard | Penaeus-monodon |
|--|----------|-----------------|
| NH out – of – plane bending | 752 | 752 |
| Ring stretching | 896 | 893 |
| CO stretching | 1026 | 1022 |
| CH ₂ bending and CH ₃ deformation | 1418 | 1421 |
| Amide II band | 1563 | 1552 |
| Amide I band | 1661 | 1643 |
| CH stretching | 2878 | 2865 |
| Symmetric CH ₃ stretching and asymmetric CH ₂ stretching | 2930 | 2921 |
| NH stretching | 3268 | 3283 |

reducing agent, DTT. This resulted in the formation of water insoluble chitosan precipitates or flocculants, and water soluble supernatants that included the deproteinization agent and any proteins that have complexed with it and the demetallization agent with the extracted metals. Adding the reducing agent enhanced the water solubilization of protein impurities by dissociating any disulfide bonds present in the proteins. The demetallization agent was mixed with the chitosan solutions under basic pH conditions to avoid the formation of chitosan-metalchelate conjugates. The degree of deacetylation (DD) of chitosan plays a significant role for determining the specific applications of chitosan.

Degree of deacetylation of the compound is 89.79% However, these higher DD values are the consequence of the high amount of protein, yielding high quality of chitosan suitable for the pharmaceutical application. Degree of deacetylation affects the-chemical, physical and biological properties of chitosan, such as adsorption, covalent linking, encapsulation. The viscosity of the pharmaceutical chitosan is very important aspect in handling as polymer for the drug delivery. The viscosity profile 304 cps of the chitosan prepared shows that it would fall under the region of most pharmaceutical products do which are suitable for designing drug delivery systems..The Prolonged decalcification time, even during 24 h, results in a very slight drop in the ash content but can cause polymer degradation the calcium carbonate which demonstrates as ash value is very low. The FT-IR studies of the chitosan from standard commercial species. The

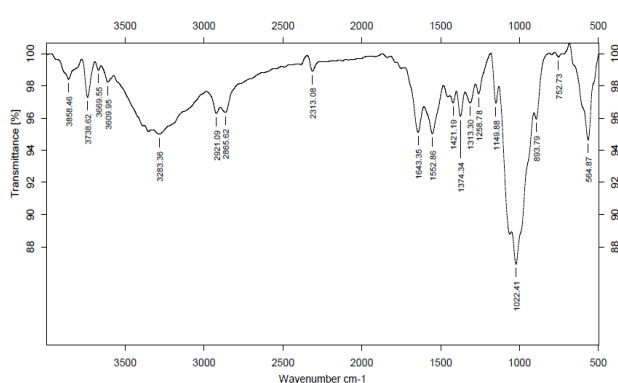


Figure 1: The FT-IR report of the standard chitosan.

major absorption band is observed between 1220 and 1020 cm^{-1} which represents the free amino group ($-\text{NH}_2$) at C2 position of glucosamine, a major group present in chitosan. Further the sample showed the absorption bands for the free amino group between 1026 and 1259 cm^{-1} when the peak at 1374 cm^{-1} represents the $-\text{C}-\text{O}$ stretching of primary alcoholic group ($-\text{CH}_2 - \text{OH}$). The absorbance bands of 3268, 2930, 2878, 1563, and 1418 cm^{-1} indicated the N-H stretching, Symmetric CH_3 stretching and asymmetric CH_2 stretching, CH stretching, $\text{C}=\text{O}$ stretching in secondary amide (amide I) and $\text{C}-\text{N}$ stretching in secondary amide (amide II), respectively. In the present study also the same absorbance bands were observed at 3283, 2921, 2865, 1643, 1552, 1421, 1022, 893 and 752 cm^{-1} which confirms the structure of chitosan from **table 2** and **figure 1 and 2**.

CONCLUSION

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 instance Chitosan used in the wound healing and scaffolds must be in the form of oligomers having low molecular weight in former case where as later ones need more proliferated structure and high molecular weight for tissue engineering. Drug delivery systems such as floating, intestinal, and other gel forms require high molecular weight with high degree of deacetylation. Simultaneously vaccine, gene, and enzyme drug delivery systems are suitable in high molecular weight chitosans which are highly deacetylated (>85%), different techniques are employed for these

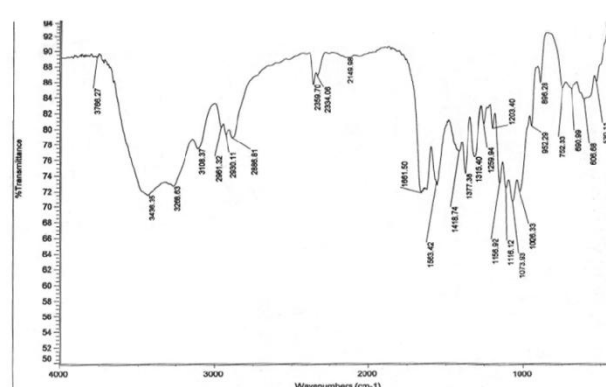


Figure 2: The FT-IR report of the chitosan extracted from the penaeusmonodon.

type of drug delivery systems such as adsorption, covalent linking, encapsulation, which require dense network, potentially charged ends and long chains respectively. Chitosan is used in design of many different types of drug carriers for various administration routes like oral, parenteral, nasal, buccal, transdermal, vaginal, topical etc. It can be formulated as nanoparticles, microspheres, membrane sponge etc. 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 biopolymer for the development of new derivatives such as Water-soluble derivatives of chitosan, Quaternarized derivatives, Carboxyalkylation, Chitosan Esters, sulfonated derivatives of chitosan, N-trimethylene chloride chitosan, Chitosan conjugates:

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