RESEARCH
O&G Forum 2024; 34 – 2s: 520 - 525

ISOLATION OF PHOSPHORYLATED CHITOSAN FROM MARINE MOLLUSES SQUID PEN AND THEIR GROWTH OF INHIBITION IN DENTAL MICROBIAL PATHOGENS

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Abstract
Introduction: The seafood processing industry produces large quantities of byproducts and discards such as heads, tails, skins, scales, viscera, backbones, and shells of marine organisms. Although these are waste residues, they still are an excellent source of lipids, proteins, pigments, and small molecules, and moreso a source of chitinous materials. One of the limitations in the use of chitin on a large-scale is its water insolubility, this is why water-soluble derivatives have been sought after.
Materials & methods : Chitosan is obtained from chitin by a process called deacetylation, and is converted into water-soluble form by phosphorylation. These chemical alterations produce a large variety of derivatives with several uses in a variety of industries.
Results: The phosphorylated chitosan derived from squid pen of *Loliolus investigatoris* produced positive results on characterization using SEM, FT-IR and XRD comparing with standard chitosan. Also the antimicrobial activity was prominent against *C. tropicolis, P. aeruginosa*, and *E. coli,* but no significant results were recorded against *S. mutans*
Conclusion: The results showed positive results in the characterization of the phosphorylated chitosan and good antimicrobial activity against the dental pathogens.
Keywords: Antimicrobial activity, Gladius, *Loliolus investigatoris*, Phosphorylated chitosan, Squid Pen

INTRODUCTION :

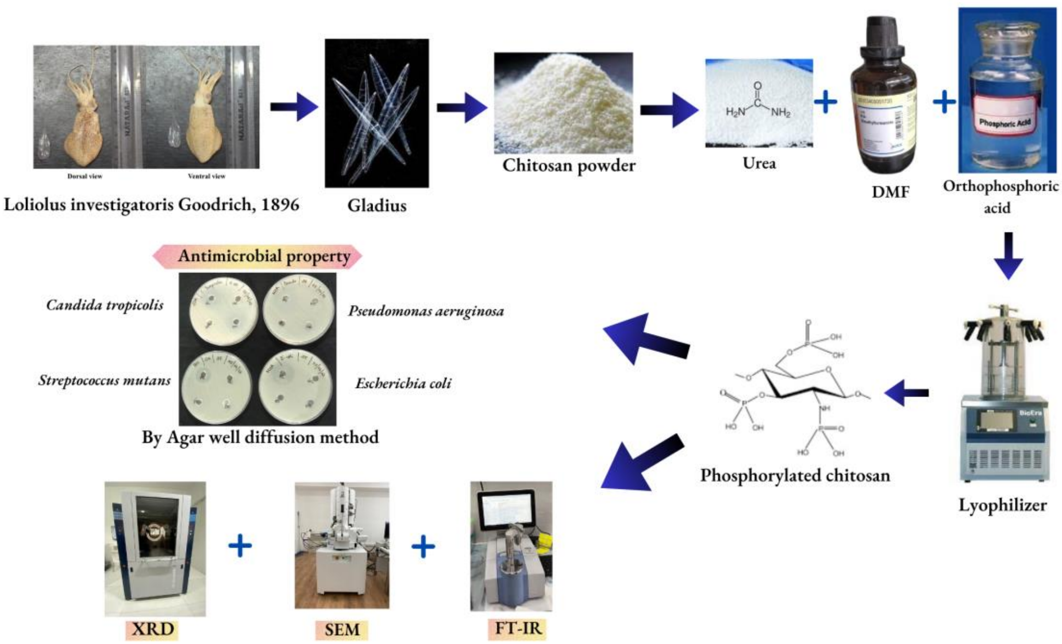
One of the biomaterials widely employed in drug delivery applications is chitosan, a cationic polysaccharide produced by the deacetylation of chitin. It consists of repeated units of -(1-4) N-acetyl glucosamine and D-glucosamine that undergo protonation at the physiological pH. Acetic acid and lactic acid are two aqueous acids where chitosan is soluble, while neutral and basic environments do not readily dissolve it. Chitosan's solubility is influenced by several factors, including molecular weight, pH, temperature, and polymer crystallinity. High deacetylation levels and low molecular weights increase solubility. (Elieh-Ali-Komi and Hamblin, 2016) Chitosan shares structural similarities with glycosaminoglycans (GAG), one of the elements of the extracellular matrix (ECM) that interact with collagen fibres and are crucial for cell-cell adhesion. After depolymerization, chitosan produces monomeric products (glucosamine), which are either metabolised by the body or eliminated from it. These bioactive chito-oligosaccharides have outstanding anti-microbial characteristics. Because of this, Chitosan is biodegradable and excellently biocompatible with practically all biological tissues. Significant osteoconductivity but little osteoinductive activity have been found in chitosan. It promotes osteoblast and mesenchymal cell proliferation as well as in vivo neovascularization. (Saravanan *et al.*, 2013)

While chitin and a little amount of chitosan are also present in the cell wall of some fungi, chitin is mostly found in the exoskeleton of crustaceans and insects. In addition, chitin-protein complex carotenoids (astaxanthin), lipids, and minerals such inorganic carbonate salt are all components of the exoskeleton of crustaceans. Chitin typically makes up 20–30% of the crustacean. Chitin, which is chemically related to glucans (80–90%), is present in fungus and insects at levels of 2-44% and 5-25%, respectively. Depending on the environment that a species grows in, this percentage changes. (Davis, 2011) Chitosan phosphorylated derivatives can be obtained in a few different ways. The significance of these derivatives originates from their remarkable biological and chemical characteristics. Additionally, they might have osteoinductive and antibacterial effects. When heated with orthophosphoric acid in N,N-dimethylformamide (DMF), chitosan can be converted to phosphorylated chitosan. (van den Broek and Boeriu, 2020)

Squids are cephalopods that are part of the Decapodifomes superorder. It may be found in benthic, burrowing in mud, sand, rocks, coral reefs, and open ocean environments. Due of their tremendous adaptability, they can relocate to areas where they can flourish. There are roughly 290 species of squid in the globe, and about 30 to 40 of them are commercially significant. They are mostly found in the Arabian Sea, the Indo-Pacific, and along the African coast to the Red Sea. There is currently a huge global demand for value-added seafood. The limbs, tentacles, and ink are all edible, while the gladius, commonly known as the pen, is the only component of the squid that cannot be consumed. (Chavan et al 2017) Loliolus has been an ill-defined genus of loliginid squid considered limited in distribution to the Indian Ocean and eastward into the Indonesian chain. L investigatoris Goodrich, 1896, was first identified from the Bay of Bengal (Sundaram et al 2004) Not only Gram-positive and Gram-negative bacteria, but also yeast and moulds, are significantly inhibited by chitosan. The utilisation of chitosan as a polysaccharide medicine has been constrained by its low solubility. The chitosan derivative had been made by adding sulphate, phosphate, carboxyl group, etc. to increase its solubility and bioactive potential. (Shanmugam, Kathiresan and Nayak, 2016)

The goal of this study was to isolate phosphorylated chitosan from the gladius of *Loliolus investigatoris*, which is frequently wasted in the food sector, and to characterise its structural characteristics and antimicrobial activity against dental microbes and fungi.

MATERIALS AND METHODS:



Collection of sample:

Gladius of squid *L. investigatoris* was obtained from a seafood processing plant at Thondi (Lat. 9°44'N; Long. 079°02' E). Gladius were packed in plastic bags and stored at -20°C before and during transportation to the laboratory. The gladius was dried, cleaned with distilled water, and then crushed in a mortar and pestle. Ortho-phosphoric acid and standard chitosan were bought from Sigma Chemical Co. (St. Louis, MO). All additional compounds were of the analytical variety.

Extraction of chitin and chitosan from gladius

Chitin was extracted from the pulverized sample by demineralization and deproteinization. The powder of gladius was treated with 2 N HCl for 24 h to remove the mineral content and then treated with 1 N NaOH at 80°C for 24 h to remove protein (Takiguchi, 1991a, Chap. 1). Chitin was deacetylated into chitosan in 40% NaOH and purifed by precipitation in 10% acetic acid (Takiguchi, 1991b, Chap. 2).

Phosphorylation of chitosan

2 grams of chitosan powder were dissolved in 30 grams of urea and 50 milliliters of DMF to produce phosphorylated chitosan. The chitosan solution was subsequently given 5.2 ml of ortho-phosphoric acid. At 150 °C, the reaction took place for one hour. The reaction mixture was precipitated, completely washed with methanol after cooling, and the residue was then redissolved in distilled water. A pH adjustment of 10-11 was made. Utilizing a dialysis membrane with a 12,000 Da MW cut-off, the solution was dialyzed for 48 hours against distilled water. In order to obtain phosphorylated chitosan, the product was lyophilized next. (Subhapradha N et al 2018)

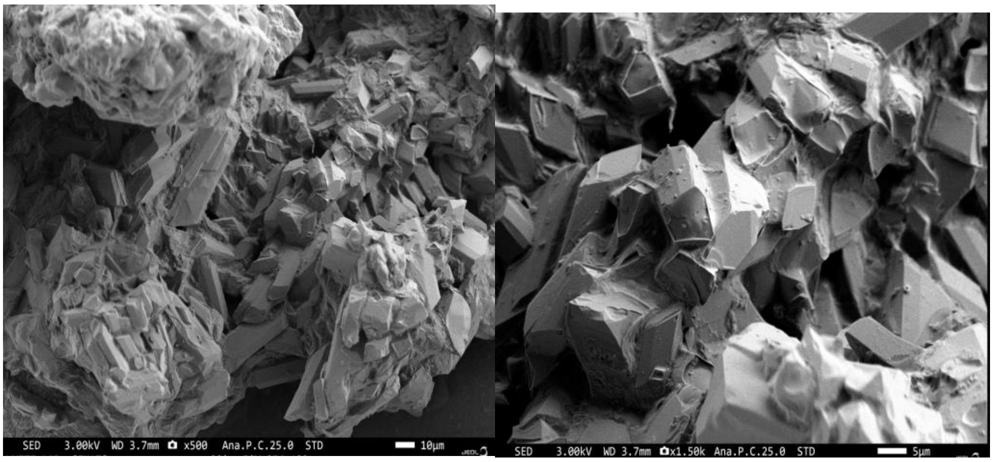
FT-IR spectral analysis

The BRUKER'S ALPHA II FT-IR Spectrometer was used for the FT-IR spectroscopy of solid samples containing both standard chitosan and phosphorylated chitosan from L. investigatoris. Salt discs (10 mm in diameter) were prepared by compressing the sample (10 mg) with 100 mg of dried potassium bromide (KBr).

The current study was carried out in Maroon Lab in Saveetha Dental College & Hospitals, Chennai for 3 months.

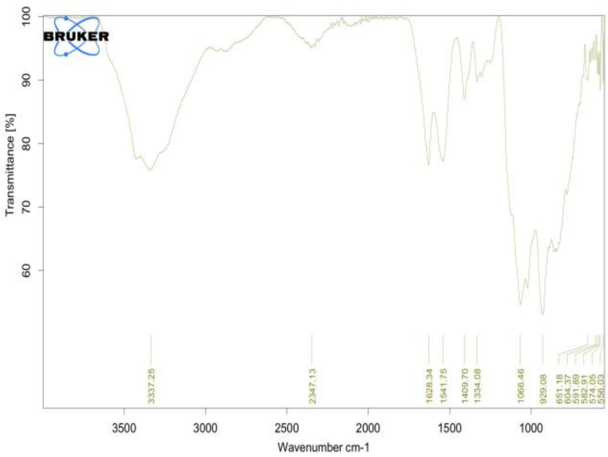
RESULTS:

Figure 1: SEM analysis



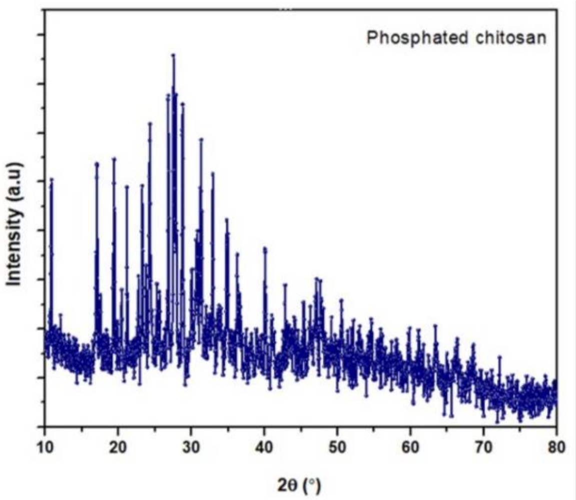
- It exhibited a cross-section of randomly oriented grains and also gave an image of the upper part of bread slice.

Figure 2: FT-IR



- The intensity of bands around 3337.25 cm−1 in the IR spectrum was due to the hydroxyl stretching vibration of the phosphorylated chitosan and as expected they were broad.
- The absorptions at 1628.34 and 1541.75 cm−1 were assigned to the stretching vibrations of the CHO and C= O bonds.

Figure 3: XRD



- It displayed two weak peaks at around 2θ of 20°and35°
- However, the peak observed for phosphorylated chitosan at 2θ = 10°disappeared and the very broad peak at 2θ = 20°became weak in phosphorylated chitosan

Figure 4: Anti-microbial activity

Table 1 :

S. No	Bacterial/Fungal Strains	Phosphorylated chitosan from the gladius of *Loliolus investigatoris*		+C (mm)	-C (mm)
		50% (mm)	100% (mm)		
1	*Candida tropicolis*	-	16±0.82	34±2.75	-
2	*Streptococcus mutans*	-	-	23±1.53	-
3	*Pseudomonas aeruginosa*	-	14±1.53	30±2.25	
4	*Escherichia coli*	-	11±0.82	25±2.75	

+Control (1. Fluconazole, 2. Streptomycin, 3. Ciprofloxacin, 4. Chloramphenicol); -Control (H_2O)

- Phosphorylated chitosan showed comparable effective results against *C. tropicolis, P. aeruginosa,* and *E. coli.*
- However, *S. mutans* has not been effectively combated.

DISCUSSION:

Annual production of fishery wastes is about millions of tons. These wastes have high biological and chemical oxygen demand, as well as pathogens, organic materials, etc., which make them extremely harmful for the environment. These wastes, however, are viewed as a potential resource with significant added value because they mostly comprise proteins, minerals, and chitin that has been deacetylated to create chitosan. (El Knidri H et al 2018) The pen, or gladius, of the squid is an internalized shell. It serves as a site of attachment for important muscle groups and as a protective barrier for the visceral organs. The pen's durability and flexibility are derived from its unique composition of chitin and protein (Messerli MA et al 2019) The overall goal of this study was not only to prepare water soluble phosphorylated chitosan from the squid pen of *L. investigatoris* but also to assess its structural characteristics and antimicrobial activity against common dental pathogens & fungi.

The structural change of chitosan after chemical treatment was cofirmed using FT-IR by comparing with standard chitosan (Figure 2). The hydroxyl stretching vibration of the phosphorylated chitosan was responsible for the broad and intense bands at 3337.25 cm−1 in the IR spectra. The stretching vibrations of the CHO and C=O bonds were attributed to the absorptions at 1628.34 and 1541.75 cm−1. (Subhapradha N et al 2018) The surface morphology and microstructure of phosphorylated chitosan was observed using a scanning electron microscope. It displayed a cross-section of grains that were randomly arranged and also provided a picture of the top of a slice of bread.

The crystalline structure of phosphorylated chitosan was analysed by x-ray diffraction (XRD) analysis. The corresponding diffract organs are shown in Figure 3. As a function of the diffraction angle 2 and the orientation of the specific materials, the intensity of the diffracted X-rays was measured. With the use of this diffraction pattern, the specimen's crystalline phases were identified, its structural properties were assessed (very accurately), and the size and orientation of crystallites (tiny crystalline regions) were all determined. (Wu D et al 2019) However, the peak seen for phosphorylated chitosan at 2 = 10° disappeared and the very broad peak at 2 = 20° became weak in phosphorylated chitosan. It showed two weak peaks at roughly 2 of 20° and 35°.

Different explanations have been put up, however the precise mechanism behind the antibacterial activity of chitosan derivatives is yet unknown. The water-soluble chitosan derivative is alleged to have increased cell membrane permeability and, as a result, broke bacterial cell membrane, allowing for the discharge of cellular contents.

(Shanmugam A et al 2016) Chitosan has been shown to have antimicrobial activity against a wide variety of bacterial strains, filamentous fungi, and yeasts. However, the degree of chitosan N-deacetylation, the molecular weight and molecular fraction of glucosamine units in the polymer chain, the pH of the chitosan solution, and the target microorganism all have a substantial role in chitosan's biological activity. (Subhapradha N et al 2013) Against *C. tropicolis, P. aeruginosa*, and *E. coli,* phosphorylated chitosan demonstrated comparably positive outcomes. However, no real progress was made against *S. mutans* (Table 1)

CONCLUSION:

The structural change of chitosan after chemical treatment was confirmed using FT-IR, and the surface morphology and microstructure of phosphorylated chitosan were observed using a scanning electron microscope. The crystalline structure of phosphorylated chitosan was analyzed using XRD, and the crystalline phases were identified. The exact mechanism behind the antibacterial activity of chitosan derivatives is yet unknown. Phosphorylated chitosan has shown positive antimicrobial activity against *C. tropicolis, P. aeruginosa*, and *E. coli,* but no significant results were recorded against *S. mutans*. However, the degree of chitosan N-deacetylation, molecular weight, molecular fraction of glucosamine units, pH of the chitosan solution, and target microorganism all play a significant role in its biological activity.

Conflict of interest: All the authors declare that there was no conflict of interest in the present study

Author Contributions: All the authors have equally contributed.

Acknowledgement: The authors would like to thank Saveetha Dental college and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha university for providing research laboratory facilities to carry out the study.

Source of funding:
The present project was funded by
- Saveetha Institute of Medical and Technical Sciences
- Saveetha Dental College and Hospital
- Saveetha University
- Aishwaryam Speciality Hospital

Ethical clearance number : Since it is an in vitro study, ethical clearance is not needed.

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