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Determination of Minimum Inhibitory Concentration for Chitosan Extracted from *Metapeuous monoceros* Against Gram Positive Bacteria, Gram Negative Bacteria and Fungi.

Md. Mahbub Hasan[1], Mehedi Hasan[1*], Momotaj Parvin[1], Rubaya Khatun[1], Ahoshan Habib[1], Toriqul Islam[1], Anika Bushra[1], Sazin Islam[1]
[1]*Department of Public Health, First Capital University of Bangladesh, Bangladesh*

Abstract: *Chitin is a polysaccharide of animal origin found abundantly in nature and characterized by a fibrous structure. It forms the basis of the main constituent of the outer skeleton of insects and crustaceans like shrimp, crabs and lobster. Chitosan has a broad antimicrobial spectrum to which gram-negative, gram-positive bacteria and fungi are highly susceptible. The activity dependence on polymeric molecular weight (MW) and degree of acetylation (DA) are described. The chitosan minimum inhibitory concentrations (MIC) are summarized from this study. The data indicate that the effectiveness of chitosan varies and is dependent on species of target microorganisms.*

Keywords: *Shrimp, chitosan, Antimicrobial Effect, polysaccharide*

I. Introduction

Chitosan is composed of a ß-1,4-linked polymer of glucosamine (2-amino-2-deoxy-ß-D-glucose) with lesser amounts of N-acetylglucosamine. Chitosan is a natural, hydrophilic, non-toxic, cationic, biodegradable and biocompatible polysaccharide used mostly in pharmaceuticals industries. It is formed by deacetylation of chitin which is present in the exoskeleton of crustaceans like crab, shrimp lobster, krill, and squid etc is a (poly-N-acetylglucosamine) (Allan, et al. 1978). The degree of deacetylation (%DA) can be determined by NMR spectroscopy, and the %DA in commercial chitosan is in the range 60-100 %. Generally, more than 50% deacetylated chitin is termed as chitosan, according to others scientist chitosan is a compound soluble in 1% acetic acid, whereas chitin is insoluble. Commercially available chitosan is mostly prepared from more than 85% deacetylated chitin, with a molecular weight between 100kDa to 1000kDa. The body does not reject chitosan as foreign invaders because of its nontoxic and non-allergenic nature. Chitosan consists a higher amount of derivatives than synthetically substituted cellulose with three unique properties- biocompatibility, biodegradability, and absorption. While soluble in acidic solution, chitosan exhibits positive charge due to the presence of primary amines on the molecule that binds protons. Chitosan consist of amino acid group with pKa value of ~6.5, although it's soluble in acidic pH to neutral solution with a charge density depending on pH and the %DA value and is positively charged which causes its bio-adhesive properties to readily binds with any negatively charged surfaces which can be attributed to the same type of ionic interactions with mucosal membrane components. (Rinaudo et al. 1999).

The maximum soluble concentration varies with different chitosan concentration, but is usually around 10-20g /L of chitosan. Chitosan solutions have good film-forming property and are therefore potentially useful in gels and coatings (Vårum, 1994). As chitosan is the second most abundant dietary fibre after cellulose and of low cost, chitosan and its derivatives have been used at least at the experimental level in a diverse range of applications. Both molecular weight and degree of deacetylation affected the film properties. Powder x-ray diffraction patterns and differential scanning calorimeter thermograms of all chitosan films indicated their amorphous state to partially crystalline state with thermal degradation temperature lower than 280-300°C.

II. Chitosan Extraction and Preparation

The shrimps (*Metapeuous Monoceros*) were collected from Khulna shrimp industry, Khulna, Bangladesh (Lat. 22.8167° N, Long. 89.5500° E) and the shell and operculum are removed from the animal. The shrimps exo-skeletons 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 exo-skeletons 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). 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 (Linden et al. 2005). 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. The exoskeletons are then further crushed to pieces of 0.5-5.0 mm using a meat tenderizer.

The shells were suspended in 4% HCl at room temperature in the ratio of 1:14(w/v). After 36 hours, the shells were quite squashy and were rinsed with water to remove acid and calcium chloride. The demineralized shells were then treated with 5% NaOH at 90°C for 24 hours with a solvent to solid ratio of 12:1(v/w). The residue was then collected and washed to neutrality in running tap water. Then it was dried in sun and the product is chitin. Removal of acetyl groups from the chitin was achieved by using 70% NaOH solution with a solid to solvent ratio of 1:14 (w/v) at room temperature for 72 hours. The mixture was stirred after some times for homogenous reaction. The resulting chitosan were washed to neutrality in running tap water and rinsed with distilled water. Then filtered and dried in sun. We know that chitosan dissolve in 1% acetic acid but chitin cannot. Now the test tube containing the most white colored 5g chitosan mixture was dissolved in 2.5% solution of acetic acid to make a 2% solution of chitosan. The solution was stirred properly and rotated thoroughly in a hand mortar to dissolve all the chitosan in it. The remaining precipitate that was observed in it was the chitin that could not dissolve in acetic acid. The obtained chitosan has to be purified to make it suitable for the pharmaceutical use. The purification process was designed in two steps:

1. Removal of insoluble with filtration
2. Re-precipitation of chitosan with 1 N NaOH

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

III. Antifungal Activity of Chitosan

Similarly to bacteria, the chitosan activity against fungus is assumed to be fungistatic rather than fungicidal with a potential to communicate regulatory changes in both the host and fungus (Raafat et al. 2008). Generally chitosan has been reported as being very effective in inhibiting spore germination, germ tube elongation and radial growth (El Ghaouth et al. 1992). Most of the This Study have been done on yeasts and moulds associated with food and plant spoilage. For these, in the presence of chitosan, several biological processes are activated in plant tissue, where chitinases are induced with action on biotrophic and necrotrophic mycoparasites, entomopathogenic fungi and vesicular arbuscular mycorrhizal fungi (Sashi et al. 1993).

The antifungal mechanism of chitosan involves cell wall morphogenesis with chitosan molecules interfering directly with fungal growth, similarly to the effects observed in bacteria cells. Microscopic observation reported that chitosan oligomers diffuse inside hyphae interfering on the enzymes activity responsible for the fungus growth. The intensity of degradation action of chitosan on fungal cell walls is also dependent upon the concentration, DA and local pH.

IV. Antibacterial Activity of Chitosan

Chitosan is antimicrobial against a wide range of target organisms. Activity varies considerable with the type of chitosan, the target organism and the environment in which it is applied. Consequently, literature reports vary somewhat and are, occasionally, contradictory. But generally speaking, yeasts and moulds are the most sensitive group, followed by Gram-positive bacteria and finally Gram-negative bacteria. Experimental work with the baker's yeast *Saccharomyces cerevisiae* showed that fermentation was halted by as little as 3.6mg L^{-1} chitosan in a buffer system. Similar powerful activity has been demonstrated against the mould *Fusarium solani*, the growth of which was prevented by 4mg L^{-1} chitosan in a liquid nutrient medium. Variation in sensitivity between closely related micoorganisms was illustrated in an experiment in which phytopathogenic fungi were screened for sensitivity to chitosan in liquid media. One Cytosporina sp. isolate was completely inhibited by 75mg L^{-1} chitosan, while a second isolate of the same genus was unaffected by 1000mg L^{-1}. There are several factors, both intrinsic and extrinsic, that affect the antimicrobial activity of chitosan. It has been demonstrated that lower molecular weight chitosans (of less than 10kDa) have greater antimicrobial activity than native chitosans. However, a degree of polymerisation of at least seven is required; lower molecular weight fractions have little or no activity. Highly deacetylated chitosans are more antimicrobial than those with a higher proportion of acetylated amino groups, due to increased solubility and higher charge density. Lower pH increases the antimicrobial activity of chitosan for much the same reasons, in addition to the 'hurdle effect' of inflicting acid stress on the target organisms. Temperature also has an effect, as - not ideally for many food applications- higher temperature (37°C) has been shown to enhance antimicrobial activity compared to refrigeration temperatures. However, the greatest single influence on antimicrobial activity is the ingredients & additives surrounding matrix. (Sekiguchi S et al. 1994)

Gram-negative bacteria appear to be very sensitive to chitosan, exhibiting increased morphological changes on treatment when compared to gram-positives (Eaton et al. 2008). The charge density on the cell surface is a determinant factor to establish the amount of adsorbed chitosan. More adsorbed chitosan would evidently result in greater changes in the structure and in the permeability of the cell membrane. That's why antibacterial mode of action is dependent upon the host microorganism.

Another mechanism is the binding of chitosan with microbial DNA, which leads to the inhibition of the mRNA and protein synthesis via the penetration of chitosan into the nuclei of the microorganisms (Hadwiger et al. 1981). In this the chitosan molecules is assumed to be able to pass through the bacterial cell wall, composed of multi-layers of cross-linked murein, and reach the plasma membrane. Observation by confocal laser scanning microscopy (Liu et al. 2001) confirmed the presence of chitosan oligomers (a chain with few number of monomer units) inside E. coli exposed to chitosan under different conditions. (Raafat et al. 2008) stated that in spite of been accepted as a possible mechanism, the probability of it occurring is rater low. The prevailing contention is that chitosan acts essentially as an outer membrane disruptor rather than as a penetrating material (Eldir et al. 2008).

The third mechanism is the chelation of metals, suppression of spore elements and binding to essential nutrients to microbial growth (Cuero et al. 1991). It is well known that chitosan has excellent metal-binding capacities where the amine groups in the chitosan molecules are responsible for the uptake of metal cations by chelation (Eaton et al. 2008). In general, such mechanism is more efficient at high pH in where positive ions are bounded to chitosan, since the amine groups are unprotonated and the electron pair on the amine nitrogen is available for donation to metal ions. A model proposed based on the system chitosan-Cu, relate the pH dependence on the proportion of available sites for interacting in polysaccharide backbone (Guibal et al. 2004). At pH < 6 the complexation involves only one NH_2 group and three hydroxyls or H_2O molecules, while at pH > 6.7 is likely to have two NH_2 involved in the complex formation. For higher pHs, i.e., 7-9, the deprotonation of hydroxyl groups are considered to occur and the predominant complexation is ruled by two $–NH_2$ and two hydroxyl groups dissociated. Similarly, in a recent model proposed by (Wang et al. 2005) the metal is arranged as an electron acceptor connected to one or more chitosan chains via $–NH_2$ and by forming bridges to hydroxyl groups.

It is unquestionable that chitosan molecules in bacteria surrounds might complex metals and blockage some essential nutrients to flow, contributing to cell death. Nevertheless, this is, evidently, not a determinant antimicrobial action since the sites available for interaction are limited and the complexation reach saturation in function of metal concentration. Influence of the Degree of Acetylation and Molecular Weight.

This Study has shown that the biological activity of chitosan depends significantly on its molecular weight (MW) and degree of acetylation (DA). Both parameters affect the antimicrobial activity of chitosan independently, though it has been suggested that the influence of the MW on the antimicrobial activity is greater than the influence of the DA.

V. Result

This Study conducted in nutrient agar on cultures of *Aspergillus fumigates* and *Aspergillus parasiticus* reveled that the percentage of fungus germination decreased with increasing the chitosan concentration in the medium. Generally the primary observed influence is on the length of the lag phase. As the inhibition process takes place, the medium shifted toward alkalinity which reduces the effectiveness of the chitosan.

Inhibition rate in order of 80% against plant fungus such as *Botrytis cinerea* and as high as 95% against *Candida albicans* and *Microsporum canis* have been, however, known to occur with low chitosan concentration (20-150 $mg.L^{-1}$)

This Study has been carried out on *Escherichia coli, Salmonella enterica, Samonella tiphymurium, Pseudomonas aeruginosa, Shigella dysenteriae, Vibrio cholerae, Enterobacter aerogenes, Bacillus cereus, Staphylococcus aureus* and *Lactobacillus brevis* proved that for lower chitosan MW (LMW), greater is the observed effect on the reducing of microorganism growth and multiplication. The size and conformation appears to be fundamental to understand the effectiveness of LMW chitosan. The mobility, attraction and ionic interaction of small chains are easier than of big ones facilitating the adoption of an extended conformation and an effective binding to the membrane surface.

The DA is determinant in the solubility and charge development, where the $–NH_2$, –OH groups in the molecule of chitosan are considered as the dominating reactive sites. Hence as the DA is reduced, higher will be the free amino groups present in chitosan and higher will be the antimicrobial effect.

Table 1: MIC (ppm) of Chitosan against Sensible organisms

Sensible organisms	MIC (ppm)
Gram negative	
Escherichia coli	20
	100
	468
	650
	1000
Salmonella enterica	2000
	3000
Samonella tiphymurium	>1000
	1500
	2000
Pseudomonas aeruginosa	>200
	1700
Shigella dysenteriae	>200
Vibrio cholerae	200
Enterobacter aerogenes	250
Gram positive	
Bacillus cereus	<1000
	1000
Staphylococcus aureus	20
	100
	>800
	700
	>1250
Lactobacillus brevis	1000
Fungi	
Aspergillus fumigatus	>2000
Aspergillus parasiticus	>2000
Botrytis cinerea	10
Candida albicans	500
	600
	>1250
Microsporum canis	1100

VI. Discussion

Several advantages of Chitosan have been found over regular type of disinfectants owing to its broad spectrum of activity. Chitosan has been observed to act more quickly on fungi than on bacteria and activity against typhoid organisms are comparable to the standard antibiotics used in clinical practice. MIC is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. MIC however is useful as a practical indicator of a primary activity against a selected pathogenic microorganism. Chitosan is a versatile material with proved antimicrobial activity. Three antibacterial mechanisms have been identified: i) the ionic surface interaction resulting in wall cell leakage; ii) the inhibition of the mRNA and protein synthesis via the penetration of chitosan into the nuclei of the microorganisms; and iii) the formation of an external barrier, chelating metals and provoking the suppression of essential nutrients to microbial growth. It is likely that all events occur simultaneously but at different intensities. The molecular weight (MW) and the degree of acetylation (DA) are also important factors in determining such activity. In general the lower the MW and the DA, the higher will be the effectiveness on reducing microorganism growth and multiplication. A study of previous work from the literature has not lead to any conclusive data as to whether the chitosan has higher activity on gram-positive or on gram-negative bacteria. On both species chitosan seems to act differently, though in both cases satisfactorily. Water soluble derivatives, which can be attained by chemical introduction of CH3 in the main chain, enhancing the chitosan applicability in a large pH range and also improve the antimicrobial activity, opening up a broad range of possibilities.

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