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THE RELATION BETWEEN CHITOSAN DEACTLATION VALUE AND INHIBITION OF *PSEUDOMONAS AEROGINOSA*

Javad Chehri
Moscow State Academy of Veterinary Medicine, Biotechnology (K.I. Skryabin), Russia
E- Mail: Javad.chehri@gmail.com

ABSTRACT

Chitin is a long-chain polymer of an N-acetylglucosamine, a derivative of glucose, and is found in many places throughout the natural world. Chitin is the most abundant natural amino polysaccharide and is estimated to be produced annually almost as much as cellulose. It has become of great interest not only as an underutilized resource, but also as a new functional material of high potential in various fields, and recent progress in chitin chemistry is quite noteworthy. The goal of this research is to investigate the diversity of antimicrobial effects of deacetylated chitosan extracted from a certain shrimp waste against clinical Pseudomonas aeroginosa. Chitosan, the most important derivative of chitin, outlining the best techniques to characterize it and the main problems encountered in its utilization. Chitosan, which is soluble in acidic aqueous media, is used in many applications. Degree of deacetylation of chitosan samples and its structure were measured by Fourier Transform Infrared Spectroscopy and Scanning electronic microscopy. In the present research chitin and chitosan were extracted from Penaeus semisulcatus waste by chemical and microbial methods. Deacetylation process of chitin was carried out in alkaline solution at different time scale. Antimicrobial activity was tested against clinical Pseudomonas aeroginosa by agar disc diffusion method. Finally, wound band was made by these compounds and antimicrobial activity was studied invitro. The result of present study confirmed the degree of deacetylation of chitosan samples up to 70% by Fourier Transform Infrared Spectroscopy method.

KEYWORDS: Chitosan, Chitin, *P. aeruginosa*

1. INTRODUCTION

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It is made by treating shrimp and other crustacean shells with the alkali sodium hydroxide.Chitosan has a number of commercial and possible biomedical uses. It can be used in agriculture as a seed treatment and biopesticide, helping plants to fight off fungal infections. In winemaking it can be used as a fining agent, also helping to prevent spoilage. In industry, it can be used in a self-healing polyurethane paint coating. In medicine, it may be useful in bandages to reduce bleeding and as an antibacterial agent; it can also be used to help deliver drugs through the skin. Brugnerotto *et al.* (2001). *Pseudomonas aeruginosa* is member of the Gamma Proteobacteria class of Bacteria. It is a Gram-negative, aerobic rod belonging to the bacterial family Pseudomonadaceae. Since the revisionist taxonomy based on conserved macromolecules (e.g. 16S ribosomal RNA) the family includes only members of the genus Pseudomonas which are cleaved into eight groups. Pseudomonas aeruginosa is the type species of its group. which contains 12 other members. Zhang *et al.* (2006)

Figure 1. *Pseudomonasaeruginosa*

Pseudomonas aeruginosa is a common Gram-negative, rod-shaped bacterium that can cause disease in plants and animals, including humans. Pseudomonas aeruginosa is an opportunistic pathogen, meaning that it exploits some break in the host defenses to initiate an infection. In fact, *Pseudomonas aeruginosa* is the epitome of an opportunistic pathogen of humans. The bacterium almost never infects uncompromised tissues, yet there is hardly any tissue that it cannot infect if the tissue defenses are compromised in some manner. It causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in patients with severe burns and in cancer and AIDS patients who are immunosuppressed. *Pseudomonas aeruginosa* has become an important cause of gram-negative infection, especially in patients with compromised host defense mechanisms. It is the most common pathogen isolated from patients who have been hospitalized longer than 1 week, and it is a frequent cause of nosocomial infections. Pseudomonal infections are complicated and can be life-threatening. Blair *et al.* (1987) Pseudomonas aeruginosa infection is a serious problem in patients hospitalized with cancer, cystic fibrosis, and burns. The case fatality rate in these patients is near 50%.

Pseudomonas aeruginosa is primarily a nosocomial pathogen. According to the CDC, the overall incidence of *P aeruginosa* infections in U.S. hospitals averages about 0.4 percent (4 per 1000 discharges), and the bacterium is the fourth most commonly-isolated nosocomial pathogen accounting for 10.1 percent of all hospital-acquired infections. Several different epidemiological studies track its occurrence as a nosocomial pathogen and indicate that antibiotic resistance is increasing in clinical isolates. is a drug resistant and opportunistic pathogen. Due to the permeability barrier in the outer membrane it is naturally resistant to many antibiotics. Baxter *et al.* (1998).Infections caused by *P. aeruginosa* are increasing both in hospitals and in general community and it has been reported as one of the principal causes of nosocomial pathogen, particularly among immuno-compromised patients Chitin with the defined structure of N-steel-β-D glucosamine is one of the most widely found organic materials, after cellulose, in nature. This material is Polysaccharide with a crystal structure whose components make up a fiber network. This structure causes the structural strength and resistance of chitin creatures. Duarte *et al.* (2002)

Chitin is usually seen in nature in a complex form with other polysaccharides and proteins. This natural polymer is very similar to Mucopolysaccharidosis such as Heparin and hyaluronic acid with very high tolerance which exists as chitin α and –β. The molecular bonds of chitin –β are weaker than α type, therefore the chitin –β has more solubility than α type. Chitin in shrimp and crab shells are mostly of α type. Type β is mainly seen in Cephalopoda. Vishu et al (2005). The most abundant natural derivative of chitin is chitosan which is formed by glucosamine. In other words, composition of glucosamine steel, chitin and glucosamine is called chitosan (4). In terms of chemical and fiber traits, chitosan is similar to existing cellulose in plants. Although it differs from plant fiber, there is the possibility of attachment to fat and its digestion in digestive system. Mahmoud *et. al*. (2007). Chitin and its derivatives have extensive application in food, cosmetic, agricultural industries and environment. The anti bacterial, anti fungus and anti viral properties of these compositions make them ideal for medical use. Chitin and its derivatives are used for separation and recycling of products, membrane separation, Chromatography columns coagulation, absorbent capsules and also in permeability of membranes and reverse osmosis pressure. It also has widespread application in papermaking, textiles, photo papers. Carboxymethyl chitin is applicable in producing methamphetamine in subcutaneous injections. chitosan coverage has been studied for facilitation of diffusion for production of various medicines such as acetamide, nicotinamide, sodium benzoate, sodium salicylate, phenobarbitone, sodium and sodium cefazolin.Sabnis and Block (1997). The existing chitin in structure of some microorganism such as phycomyces spp, mucur spp, aspergillus oryzae is a suitable candidate for use in wound dressing. Salts such as chitosan, chitosan lactate and pyrrolidinone methyl chitosan are the best dressing for wounds. The existing chitin in these funguses has applications for increasing proliferation of human F_{1000} fibroblasts and production of a matrix for gradual recovery of skin swellings. Chitin and it derivatives are an effective factor in trapping metals which are responsible for occurance of many allergies. It is therefore an effective and excellent substance for sensitive and allergic skins. These natural polymers are widely used in protectors for skin and hair, moisturisers, creams for hands, face and body, bath lotions, powder cream, nail varnish and tooth paste . Khanafari (2008). In this regard, the aim of this research is to determine the level of chitin deacetylation and extracted chitosan, using chemical and microbial methods, from skin, head and carapace of the dominant shrimp Penaeus semisculcatus, changing this level by chemical methods and determining antibacterial properties of various percentages of deacetylae compounds is one of the many applications of this valuable compound.

2. MATERIALS AND METHODS

Sample:

Wastage of skin, head and carapace of the dominant shrimp *Penaeus semisculcatus* around Caspian Sea shore were used. Shrimp waste is separated from the meat by workers at factories producing canned shrimp food which is then transferred to labs on ice, where they are placed in an oven for 24 hours at a temperature of 39^{O}C until it is completely dried. The waste material is then turned into powder and kept in temperature of -25^{O}C in dark sealed jars until ready t use.

25ml (molar 2) caustic soda is added to powder from the shrimp waste and is incubated in temperature of 30^{O}C for 20 minutes to deproteinise the sample. This mixture is then rotated at 2000rpm for 15 minutes and the surface lye is separated and thrown away. The resulting sediment is washed several times with distilled water. To purify extracted chitin, the dried shrimp shell is deproteinised using 1.5% Sodium hydroxide solution with a ratio of in temperature of 90^{O}C for 2 hours. The resulting lye and sediment is then washed away. To extract chitosan from the sediment, the above mixture is exposed to acetic acid 10% with a ratio of 40:1(VW) in 60^{O}C for 6 hours and then crude and pure chitin was separated by 4000rpm rotary. The zincoid phase PH which contains soluble chitosan in acetic acid is taken to about 9 using caustic acid. Here, chitosan becomes suspended in solution and then settled.

Strain Lactobacillus plantarum PTCC is produced by Iran Scientific Research Organisation. For microbial extraction of chitin and chitosan, a 15% culture of shrimp waste powder was prepared in distilled water and iron nitrate was added at a rate of 2% to maximise chitin extraction. The above medium was then autoclaved in temperature of 125^{O}C and pressure of 1.4 atmosphere for 20 minutes. After gradual cooling of flasks containing shrimp culture in lab conditions, a 15% ratio of inoculated culture Lactobacillus plantarum PTCC (1188), which were cultured in MRS broth (meat extract 8g, peptone extracted from casein 10g, yeast extract 4g, polysorbate 85 1g, glucose 10g, Di-ammonium hydrogen citrate 2g, sodium citrate 5g, magnesium sulfate 0.2g, manganese sulfate 0.04g, distilled water 1000ml) and showed an absorption of 0.8-1 (5 x 10^{8} CFU/ml) at a wavelength of 600nm, was added. Mediums were incubated for a week at a temperature of 30^{O}C. After this period, the medium was rotated for 15 minutes at a rate of 2000rpm. The supernatant was discharged, and the resulting sediment was washed with distilled water or MRS broth. The above chemical method was used to remove the proteins and minerals from shrimp shell. (Domard and Rinaudo, 1983).

Preparation of dastylh derivative:

Chitin extracted from shrimp waste by chemical and microbial methods was mixed with sodium hydroxide 45% with a ratio of 1:10. The above samples were transferred to water bath (bain-marie) at 85^{O}C with a time period of 20, 25 and 35 minutes and finally 10 hours. Jung *et al.* (2005)

The samples were then cooled by being placed in cold water for 15mins and rotated at a 5000rpm speed. The zincoid phase was eliminated and the sediment was washed with 70% ethanol until neutralised. In the end, the samples were washed with absolute ethanol for the purpose of dewatering and were placed in the oven at a temperature of 60^{O}C for 24hrs to dry and then transferred to desiccators.

Anti-microbial quality of deacetylate components:

The amount of 0.2g of the above samples was added to 1ml mixture of Hydrochloric acid, In order to survey the anti microbial qualities of chitosan's deacetylated derivatives, aquafortis with a ration of 2:3 and it was kept at a temperature of 100^{O}C until it was dissolved.

Hospital resistant strain sample Pseudomonas aeroginosa was acquired from children's medical centre hospital. The resulting solution was then inseminated into blank disks. After drying of the discs at room tempretaure, dry weight of the absorbed sample on each disk was determined.

The sample was taken from the endotracheal tube of a newly born baby. This separated Pseudomonas sample, showed a high resistant toward antibiotics. Suspensions from the above bacteria were produced in Mueller Hinton broth medium and its tiff was compared with a McFarland 0.5 control. Then, 1ml of the above inoculated culture was added separately to agar Mueller Hinton medium and compact culture was produced using antibiogram method. The produced chitosan disks were then placed on the above inoculated culture at a distance. After incubating the samples

for 24hrs at 39^{O}C, zone for growth inhibition for each disk was measured with culture line and noted down. In order to avoid the interference of the said chemical solvents' effect, the control disk sample for each solvent and their mixtures was also considered at each stage. To compare the antimicrobial effects of the above components, two bacteria E. coli as gram-negative index and staphylococcus aureus as gram-positive index were used. Survey of antimicrobial effect of increasing concentration of deacetylated chitosan: To obtain stronger antimicrobial effects, increasing percentages with three times density was produced from deacetylated chitosan powder using above method and their antimicrobial effects were reviewed and compared.

Bandage from deacetylated chitosan:
Since one of the main problems in burns units are secondary hospital bacterial infections, specially Pseudomonas aeroginosa, and on the other hand, applying ordinary bandage for such patients causes adherence to the burnt parts, we examined the antimicrobial effect of chitosan bands on medium containing the above bacteria. (Felse and Panda ,1999). For this purpose, after turning deacetylated chitosan powder into dough, the dough was placed on the bandage and after infecting the agar Mueller Hinton medium with Pseudomonas aeroginosa bacteria using the said method, the bandage was placed on the medium and was incubated at 39^{O}C. in order to compare, the control medium without the bandage was also considered.
Statistical analysis:

In order to survey the difference in the level of growth inhibition of extracted deacetylated chitosan components on control bacteria, in constant environmental parameters, the SPSS version 18 and ANOVA test were employed and the significance coefficient of parameters was considered at P<0.05. Jahanshahi *et al.* (2010, 2011a,b,c), Karimi Takalo *et al.* (2013), Nawaser *et al.* (2014).

3. RESULTS
Results of analysis of the elements by the electron microscope showed a reduction in chitosan pore diameter in the presence of calcium ion. Also, the results of chitosan deacetylation showed the formation of chitosan membranes with pore diameter of 0.075-0.362 micron in comparison with the chitin sample using the electron microscope. By heating the chitin sample, the number of bands related to C-H (Alkenes) components at position 675-1100 and 1350-1380 cm^{-1}were observed in chemical and microbial chitosan samples, which the control sample lacked. On the other hand, by increasing heating time from 15mins to 10hrs, the number of these bands initially lowered and then in the 10hr sample became similar to the 15min sample. Also, the appearance of band related to C=C (Alkynes) components at position 2130-2280 cm^{-1} was observed in microbial sample with the increase of temperature which was lacked by the chemical and control samples (except for the sample with 35 min heating.

By heating chitin sample, the number of bands relating to C-H components was observed at position cm^{-1} 675-1100 and 1350-1390 in both chemical and microbial chitosan samples, with the control sample lacked. On the other hand, with the increase in the time of heating from 20 minutes to 10 hours, these bands were initially reduced and then in the 10 hour sample, became similar with the 20 minutes sample. The results of chitosan deacetylation also showed formation of chitosan membranes with pore diameter of 0.067-0.252 micron in comparison with chitin sample under the electron microscope. Results of analysis of elements by electron microscope showed reduction of chitosan pores in the presence of calcium ion in the medium. Results obtained from evaluation of antimicrobial effects of extracted deacetylate components, showed the formation of growth inhibition diameter on hospital bacteria Pseudomonas aeroginosa in the highest condition 15mm and the lowest condition at 11mm. These results were calculated on the basis of 3mg of deacetylate powder absorbed on each disk. Results of statistical test to review the level of antimicrobial effect of chitosan deacetylate derivatives at growth inhibition diameter, did not show a significant difference at P<0.05 between methods of microbial and chemical extraction after heating in various times. The minimum growth inhibition diameter is 15mm and is related to chemical and microbial extraction of chitosan with a 10hr heating on a control bacteria E.coli and a maximum 19mm related to microbial extraction with 15min heating for 10hrs on a control bacteria S. aureus. Statistical results show a significant difference between formation of growth inhibition diameter in accordance with time of heating of chitosan deacetylate derivatives and the type of control bacteria at P<0.05.

Regardless of heating time, maximum diameter of growth inhibition on bacteria gram-positive S. aureus is seen with average diameter of minimum 10 and maximum 20mm. When the level of effective substance on disk is increased three

folds , this bacteria's growth inhibition increased from 20mm to 25mm.
Results obtained from producing chitosan dough and the bandage showed that as long as this bandage is placed on inoculated culture medium with Pseudomonas aeroginosa bacteria, it prevents the growth of the said bacteria (results of this test were surveyed after a week of incubation). Whilst, in the control culture medium (without bandage) bacteria completely grew after 24hrs.

4. DISCUSSION

Chitin is the most abundant natural amino polysaccharide and is estimated to be produced annually almost as much as cellulose. It has become of great interest not only as an underutilized resource, but also as a new functional material of high potential in various fields, and recent progress in chitin chemistry is quite noteworthy. The goal of this research is to investigate the diversity of antimicrobial effects of deacetylated chitosan extracted from a certain shrimp waste against clinical Pseudomonas aeroginosa. Chitosan, the most important derivative of chitin, outlining the best techniques to characterize it and the main problems encountered in its utilization. Chitosan, which is soluble in acidic aqueous media, is used in many applications.

Using FT-IR method to determine the level of chitosan deacetylation was first conducted by Moore and Roberts. This method had many advantages: because it was quick and needn't desolve the chitosan sample in solvent. Chitosan has natural absorption. Reports show that chitosan's absorption reduces with increase of deacetylation.
Results obtained during this research showed that production of deacetylate components of chitin extracted from skin, head and carapace of the dominant shrimp Penaeus semisculcatus, in comparison with control sample (chitin) after preparation, were 70% and 30% respectively. This method caused a 35% reduction of chitosan samples' acetylation.

However, the time period of heating did not cause much reduction in the level of deacetylation. So much so, that in the first 15mins of heating, the level of deacetylation showed a 40% reduction in relation to control sample which did not change much until the end of the 10hr heating period. With the increase of heating period for deacetylation of chitosan sample, the pore diameter decreased from 0.5-1micron (in control sample) to 0.052-0.35micron. In other words, deacetylation increase caused the pores to close. It is showed that increasing the time of heating for the purpose of creating deacetylate components from the crab shell does not cause tangible effect in increasing the deacetylation percentage. Zhang et al (2006) research reported the diameter of membrane pores of chitosan as 0.2-60micometer and the average diameter as 0.57-13.554mm. Various reports have been presented regarding the production of chitin membranes or chitosan membrane with various pore diameters. One of the most common methods of producing these membranes is using the lyophilisation strategy which is an expensive method. Chitin and chitosan pored membranes produced by this method are used in tissue culture engineering, producing wound bandage and drug delivery carriers.

Chow and Khor used the IBP (Internal Bubbling Process) to produce chitin matrix with large pore diameter (100-1000 micrometer). Felse and Panda (1999). Produced membranes from chitin and chitosan with large pore diameters with pore size control using silica particles. In this suspension method, silica particles with the desired size are added to chitosan acidic solution. Sabnis and Block (1997) used chitosan/aluminum composite for eliminating divalent copper from polluted waters. The deacetylation method used in this research can also be a suitable and cheap method for acquiring chitosan and chitin membrane in various sizes.

With threefold increase of affective substance on disk, the growth inhibition halo on hospital Pseudomonas aeroginosa bacteria, a 50% increase was shown.

In analysis of elements using electron microscope, a significant correlation was observed between presence of calcium ion in the sample and number and size of pores. In the chemical and microbial methods of extracting chitin and its deacetylation using the above method, in the electron graphs of element analysis, in samples where calcium ion is not observed, an increase of numbers and sizes of pores is observed and contrary to this, in graphs where presence of calcium ion is verified, pores have reduced or closed. Similar linear relationship was not observed between increasing heating period (deacetylation level) with growth inhibition halo diameter on surveyed microorganism.

Table 1: Comparison of type of extracted chitosan components by microbial and chemical methods
Type of composition and the intensity of its absorption according to sample

Chitin
Chitosan, microbial extraction 20 mins heating
Chitosan, microbial extraction 25 mins heating
Chitosan, microbial extraction 35 mins heating
Chitosan, microbial extraction 10 hrs heating
Chitosan, chemical extraction 20 mins heating
Chitosan, chemical extraction 25 mins heating
Chitosan, chemical extraction 35 mins heating
Chitosan, chemical extraction 10 hrs heating

Table 2: Antimicrobial affect of chitosan deacetylate extracts according to growth inhibition diameter (mm). Growth inhibition halo diameter (mm) / microorganism

	20mins		25mins		35mins		10hrs	
	mic	chem	mic	chem	mic	chem	mic	chem.
E. coli	12	15	20	11	26	27	31	34
S. aureus	22	13	24	16	27	28	12	23
P.sp	16	15	20	31	16	23	32	30

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