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Reviewer's Name:

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- Method, Result & Conclusion should be in past tense. Please check all grammar. it needs professional proof reading.
- write the structure of MCS
- add discussion in DS & solubility test.
- In the last paragraf of result & discussion, there is no prove that MCS can apply for this purpose. should be remove or modify the sentence.

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AIP

Synthesis and Evaluation Antibacterial Activity of Phosphate Buffer Solution (pH 7, 4) - Soluble Acylated Chitosan Derivative.

Abstract The N-maleoyl chitosan (N-MCS) is a chemically modified derivative of the biopolymer chitosan was synthesized through acylation reaction between chitosan with maleic anhydride acid. The resulting N-MCS was characterized by FT-IR spectroscopy and its antibacterial activity evaluated against *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) bacterial with the Agar diffusion method in the Muller Hinton Agar. Results obtained MCS soluble in a phosphate buffer solution (PBS, pH 7.4) with a concentration of 2 % (w/v), and did not show any antibacterial activity against both test bacteria. However, with the formation of chitosan derivative soluble in the physiological medium of PBS, pH 7.4 will expand its utilization in the formation of hydrogels for biomedical applications such as drug delivery.

Key words: PBS soluble -N-maleoyl chitosan, antibacterial, acylation reaction.

INTRODUCTION

Chitosan (CS) is one of the most important biopolymers that can be readily obtained from natural resources and is one of the derivatives of chitin[1] comprising the β- (1,4) -2-amino-2-deoxy-D-glucopyranose (GlcN) includes a small amount of residual N-acetyl-D-glucosamine (GlcNAc)[2,3,4,5,6] and is the second abundant natural polysaccharide after cellulose.[2,7]

Chitosan attracts the attention of Scientists in recent years because of their special properties, biodegradability, biocompatibility, bioadhesivity, non-toxicity and useful antimicrobial activity in areas such as biotechnology, pharmaceuticals, cosmetics, agriculture, food science, the environment and textiles.[2,6] Because of its high biodegradability and non-toxicity to humans, chitosan is widely used as either its own antimicrobial agent or in combination with other natural polymers.[4,9,10]

Chitosan is soluble in aqueous acid medium[11] including inorganic acids such as HCl and organic acids such as methanoic acid, ethanoic acid, oxalic acid, and lactic acid[5] and antimicrobial activity of chitosan against various bacteria and fungi derived from its natural polycationic nature.[4,9,11] Molecular weight and deacetylation degree are the main parameters that determine solubility and physical-chemical properties, in addition their effectiveness depends on the concentration.[12]

Several mechanisms that explain the antimicrobial activity of chitosan have been postulated. The most acceptable mechanism is the interaction between the positive charge of the chitosan molecule and the negative charge of the microbial cell membrane. This interaction is mediated through electrostatic forces between protonated $NH_3^{\oplus}$ from chitosan and electronegative charges on microbial cell surfaces,[12,13] resulting in a double disturbance: first by promoting changes in membrane wall permeability properties, resulting in an internal osmotic imbalance and consequently inhibiting growth microorganisms; and secondly by peptidoglycan hydrolysis on the walls of microorganisms, causing leakage of intracellular electrolytes such as potassium ions, and other molecular protein elements (eg, proteins, nucleic acids, glucose, and lactate dehydrogenation). As such a mechanism is based on electrostatic interactions, the greater the number of cationization groups, the higher the antimicrobial activity.[13] Consequently, it is expected that polymers with higher acidity levels result in better antimicrobial activity.[12]

Chitosan activity as an antimicrobial agent has been observed in numerous studies and most of its ability to inhibit microbial growth observed in acidic medium, where the polymer dissolves and carries a net positive charge.[14] Similarly, the antibacterial activity of chitosan against E. coli and S.aureus has been were investigated in the presence of the addition of acetic acid, lactic acid, and citric acid increased inhibitory activity while with NaCl activity slightly decreased in broth culture medium containing 100 ppm chitosan (Mw 3,000).[15] The activity is limited to pH below pKa (about pH 6.5), where the kiotosan begins to lose its cationic properties and its solubility properties become less or less soluble.[4,9,10,15] is a deficiency that can limit the application and study of its bioactophysics in the wider neutral and physiological conditions.[14,17] So the solubility of chitosan in water is an important factor for wider applications so that many researchers focus on making water-soluble derivatives over a wide range of pH (Lim & Hudson, 2003).[4,13]

To improve solubility, physicochemical properties and bioactivity, several chemical modifications of CS have been reported in recent decades. Chitosan contains three types of functional nucleophilic groups of NH_2 C-2, C-3 secondary OH groups and C-6 primary OH groups.[14] and virtually all CS derivatives are synthesized through such functional modification, as well as substituted pyridyl CS[14,16] or Cs carboxymethyl, ammonium quaternary salts -CS, (QAS-AS)[9]. Some other derivatives with high water solubility have been prepared such as quaternary ammonium salt, N- (sulfate) chitosan, O- (methylenephosphonic) chitosan and N, O- (succinyl) chitosan. Chitosan derivatives contain carboxyl groups as well as N, O- (carboxyalkyl and aryl) chitosans, N, N- (dicarboxyethyl) chitosan and N- (carboxyacyl) chitosans.[4]

In an attempt to increase the solubility of chitosan in phosphate buffer solution (pH 7.4), in this study will be synthesized N-maleoil chitosan (MCS), through the opening of the maleoil group into the N-terminal chitosan of the glucosamine unit by using maleoil anhydride and further investigating its antimicrobial activity for further application in the formation of hydrogels as drug carriers or other biomedical applications.

EXPERIMENTAL

Materials and Instruments

The materials used in this research are, Chitosan, DD 85%, Mw.4 x 10^5, (Sigma-Aldrich), maleic anhydride, glacial acetic acid, methanol pa, ethanol pa, acetone pa, NaOH, NaCl, HCl pa, Na2HPO4, NaH2PO4, phenopthalein, methyl orange, hikdroksilamin hydrochloride, all products of E.erck, aquadest, pH universal paper, nutrient agar (NA), Mueller Hinton agar (MHA), E.Coli and S.aureus cultures obtained from laboratory of Microbiology, Department of Biology, University of Sumatera Utara. All chemicals are used without further purification.

Instrument are, vacuum oven (Fisons), hotplate / magnetic stirrer, magnetic bar, thermometer, pH meter (Hanna), spectrophotometer (UV-1800 Shimadzu), FT-IR spectrophotometer, biuret , autoclave, stirring rod, bunsen, petri dish, ose needle, slipper, cotton, micro pipette, refrigerator, incubator, vortex, and other glassware.

Synthesis of N-Maleoil Chitosan (MCS)

MCS is synthesized according to the procedure reported by the previous researcher [5, 18,19,20] with minor modifications; 1.0 g of chitosan is introduced into a three-neck flask and dissolved with 100 mL (2%, v/v) acetic acid solution followed by the addition of 100 mL of methanol to further homogenize and dilute the chitosan solution. In a glass beaker diluted about 5.9 g of maleic acid anhydride to 50 ml of acetone, then added dropwise to the chitosan solution while stirring at 60 ° C. Stirring the reaction mixture at 60 °C is continued for 24 hours. After cooling of the reaction product was carried out a settling process by adjusting the pH of the mixed solution to about 7-8 with the addition of NaOH 1 N. The white precipitate (MCS) formed subsequently was filtered and washed several times with ethanol to purify the product. Then dried in a vacuum oven with a temperature of 40 °C for 24 hours just kept on the desiccator until it is used.The occurrence of chitosan maleoil formation reactions is verified by condensation between amino groups of chitosan and electrophilic carbonyl groups of maleic acid anhydride, forming amide bonds by opening anhydride ring.

Determination of Degree of Substitution (DS)

Determination The substitution degree of the MSC was determined in accordance with the method reported Bashir, et al,[5]. 0.1 g NSC dissolved in 20 ml distilled water, the pH of this solution was adjusted to 2 with the addition of 0.1 N HCl. with a solution of 0.1 M NaOH. DS is calculated as stated in Equation (1):

$$DS = \frac{177 \times A}{mMCS - 100 \times A} \qquad (1)$$

Where, VNaOH and CNaOH are the volumes and concentrations of NaOH,
mNSC is the mass of NSC. 177 and 100 are the molecular weight of each unit of glucosamine and maleoyl group.

Solubility test

The solubility test of chitosan and derivatives of N- (4-carboxybutyroyl) chitosa examined in water and phosphate buffer solution 0,1N. The samples were immersed in each solvent at a concentration of 20 mg/mL and the solubility was checked after being allowed to stand for 24 hours at room temperature.[4]

Determination of antimicrobial activity.

Determination of antibacterial activity of chitosan and its derivative to E.coli and S.aureus was investigated by well diffusion method. Acetic acid solution 2% w/v, phosphate buffer solution solution pH 7,4 also tested its activity as comparison. A well with a diameter of 6 mm was prepared on agar medium which had been inoculated by E.coli and S.aureus using a sterile metallic drill, then inserted the sample to be tested for activity into the well and observed the clear zone formed after 24 hours incubation. The resulting clear zone shows the inhibitory power of the test sample on E.coli and S.aureus and is measured using a sliding (mm)

RESULTS AND DISCUSSION

Synthesis of Maleoil Chitosan and its characterization by FT-IR spectroscopy.

The reaction of chitosan maleoil formation takes place through the acylation process between the amino group of chitosan and electrophilic carbonyl group from maleic acid anhydrid, forming the amide bond. with the opening of the anhydride ring. Chitosan and N, the chitosan maleoil which is a chitosan derivative N, is characterized by FT-IR spectroscopy and the spectrum is presented in Figures 1 and 2 respectively.

Figure 1 shows the FT-IR spectrum of chitosan. The widespread absorption peak around 3448 cm^{-1} is associated with vibration stretching -NH and -OH, as well as the hydrogen bonds of inter-and extra-molecular chitosan molecules. Then there is a weak absorption at 2931 cm^{-1} is associated with CH-chitosan stretching.[2] Characteristic peak at 1651 cm^{-1} (Amide I), 1558 cm^{-1} (-NH_2 bending of amino group), and 1396 cm^{-1} (Amide III). The 1064 and 1033 cm^{-1} absorption peaks (CO stretching vibrations) are characteristic of the sugar structure. In the FT-IR spectrum of maleoyl chitosan (Figure 2) shows a weak new peak at 1165 cm^{-1}, indicating the overlap of vibration stretching the -NH and -CN groups. Compared with spekytra chitosan, maleoil chitosan showed a new absorption peak in 1712 cm^{-1} that could be attributed to an acylation reaction in the NH_2 group of chitosan. The 1319 cm^{-1} peak for the OH group on chitosan to 1317 cm^{-1} shows a slight shift to 1311 cm^{-1} in maleoyl chitosan. This result shows that the action of the succinyl derivative occurs at the N-position[21] and the appearance of peak at 825 cm^{-1} are typical uptake of C = C groups of maleoyl (19) So based on the characterization with FT-IR it can be that maleoyl chitosan has been formed.

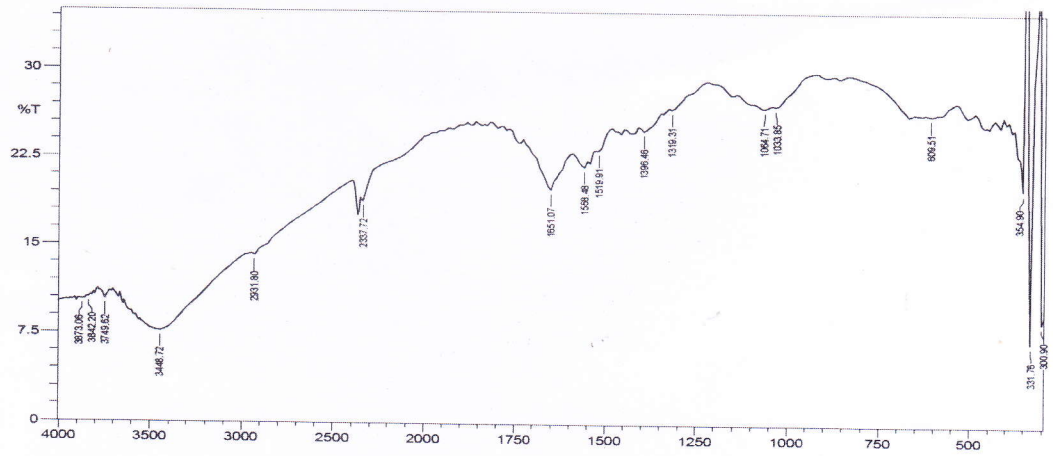

Figure 1. Spectrum FT-IR chitosan .

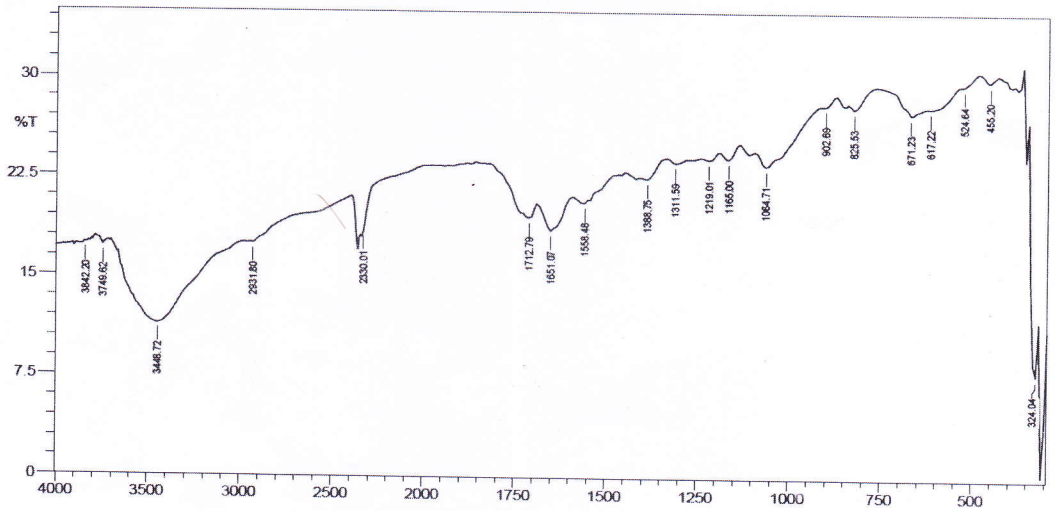

Figure 2. Spectrum FT-IR maleoyl chitosan .

Degree of substitution (DS)

The degree of substitution refers to the average number of functional group replacements by functional other clusters per repeating unit. In this study, based on the titration method the degree of substitution (DS) was found to be 0.66.

Solubility test.

The solubility of chitosan and its derivatives N-maloil chitosan was evaluated in phosphate buffer pH 7.4 solution. The observed data show that chitosan does not dissolve in the medium and its derivatives dissolve completely. This refers to the presence of carboxyl groups in the resulting molecule.

Test antimjcrobial activity.

Test results of antimicrobial activity of control solution (2% w/v chitosan, 2% v/v acetic acid and 0.1 N phosphate buffer pH 7,4), and MCS solution in phosphate buffer solution pH 7.4 were show in Table 1 below.

Table 1. Antimicrobial activity against Echerichia.*Coli* and Staphylococus.*aureus*

Bakteri	Sample solution		Inhibition zone (mm)
Escherichia coli	Control	Acetic acid	7,00
		Chitosan solution	13,10
		Phosphate buffer pH 7,4	-
	Sample	MCS	-
Staphilococcus auerus	Control	Acetic acid	19,15
		Chitosan solution	19,40
		Phosphate buffer pH 7,4	-
	Sample	MCS	-

Based on the results of the study as presented in Table 1 shows that acetic acid showed antimicrobial activity against both E.Coli and S.aureus bacteria respectively 7.00 and 19.15 mmm. These results also correspond to studies conducted by Hong, et al, 2014, that some organic acids such as acetic acid, citric acid, and lactic acid inhibit the growth of both bacteria and acetic acid has the strongest effect. Likewise, 2% chitosan solution had antimicrobial activity against both types of bacteria ie 13.1 mm for E.coli and 19.4 mm for S.aureus respectively. This result indicated that chitosan dissolved in 2% acetic acid showed that their activity was stronger inhibiting bacterial growth compared to 2% acetic acid solution.

There are several suggested mechanisms to explain this activity and the most acceptable is the interaction between the positive charge of chitosan molecule and the negative charge of microbial cell membrane. This interaction is mediated through electrostatic forces between protonated NH_3^+ from chitosan and electronegative charges on the microbial cell surface by binding and disrupting normal cell membrane function, for example by promoting leakage of intracellular components and also by inhibiting the transport of nutrients into cells causing bacteria to die. From the total samples tested its activity showed that its anti-microbial properties are stronger against S.aureus bacteria compared with E. coli.[13,16]

The maleoyl chitosan (MCS), a water-soluble chitosan derivative obtained from chitosan acetylation with maleic anhydride showed no anti-microbial activity against both bacteria. This may be due to MCS being dissolved in a phosphate buffer solution with a physiological pH 7.4. So the N atom as an element that contributes to the strength of antimiral activity of chitosan and its derivatives does not undergo protonation and does not carry cationic charge at physiological pH[9] , and the concentration of MCS solution used is 20 mg/ml which is also in accordance with previous research, N-carboxylic acid chitosan has no antibacterial activity at concentrations up to 20 mg/mL [22] and some studies also suggest that the activity of some chitosan derivatives such as NCS show is not activity at all. The N-acyl derivatives, N, Succinil Chitosan studied by Fan and team [23] have no antibacterial activity potential due to decreased load density compared with chitosan as well as studies conducted by Aziz and colleagues [24] who found that no test bacteria (Gram-positive and Gram-negative bacteria) were susceptible to hydrogel N, Succinil Chitosan (NCS). This is due to the reduction of positive charge on the amino group, and thus, NSC can not interact electrostatically with negative charges on the bacterial cell wall.[5]

However, by obtaining MCS as a chitosan derivative dissolved in a phosphate buffer solution pH 7.4 will expand its application in the pharmaceutical field such as for further use as a hydrogel-forming agent crosslinked with aldehyde groups of other natural polysaccharides in situ on pH phosphate buffer pH 7,4 medium which act as antimicrobial as well as antimicrobial agent delivery for controlled release in the stomach.

CONCLUSION

Based on the result of the research, it can be concluded that chitosan maleoil (MCS) which is derived chitosan derivatives has been successfully synthesized, found its substitution degree (DS) 0,66 and soluble completely in phosphate buffer ph phosphate 7,4 (concentration 20 mg / ml). The antimicrobial activity test showed no similar activity to the two test bacteria in contrast to chitosan showing activity against both bacteria at the same concentration. However, by obtaining MCS as a chitosan derivative dissolved in a phosphate buffer solution pH 7.4 will expand its application in the pharmaceutical field for use as a crosslinked hydrogel-forming agent in situ in a phosphate buffer solution of pH 7.4 with an aldehyde group of natural polysaccharides others that act as antimicrobials or as antimicrobial agents for controlled release in the stomach.

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Page	Row	Written	Should be
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1057-2	8	kiotosan	chitosan
1057-2	21	maleoil	maleoyl
1057-2	27	Na2HPO4, NaH_2PO4	Na_2HPO_4, NaH_2PO_4
1057-2	28	E.erck	E.merck
1057-2	29	E.Coli and S.aureus	*E.coli* and *S.aureus*
1057-3	3,6	NCS	MCS
1057-3	8	chitosa	chitosan
1057-3	20	chitosan maleoil	Maleoyl chitosan
1057-5	1	maloil	maleoyl

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