Synthesis and Characterization of β-Poly(glucose-amine)-N-(2,3-dihydroxypropyl) Derivatives as Medical Care and Biological Joint Material. Family 2. Tri or Tetra-Sulfated β-Chitosan

Ryu Ga Youn,[1] *Ryu Soung Ryual,**[1] *Jang Hang Doung,*[2] *Jo Byeong Uk*[3]

[1] Department of Chemical Engineering, Dae Bul University, Young-am gun, Chonnam, 526-702, Korea
E-mail: ryusr@mail.daebul.ac.kr
[2] Department of Chemical Engineering, Seoul National university of Technology, Nowaun Gu, Seoul, 139-743, Korea
[3] Department of Chemical Enginerring, Chosun University, Kwang Ju, Chonnam, 501-759, Korea

Summary: Chitin is a natural polysaccharide by N-acetyl-D-glucosamine units β(1-4) linked. In the present work a chitosan with DA 56% and Mv 80.000 g/mol will be employed. Several techniques to obtain sulphate derivatives of chitin and to chitosan have been proposed due to the interesting biological and chemical properties of such as heparin compounds. Among others it could be mentioned their antibactericidal and metal chelating properties. In the present work a new sulfated derivatives of chitosan with potential growth regulator properties were obtained. The reaction was carried out in heterogeneous media using a commercial growth regulator -SO_3H as sulfating agent. All derivatives were characterized by several spectroscopic techniques. In IR spectra the bands at 1240 cm^{-1}, (S=O stretching) and 860 cm^{-1}, corresponding to S-O-C symmetric stretching, are characteristic of sulphates groups. In ^{1}H-NMR spectra the appearance of functional group signal confirm its structure.

Keywords: β-chitosan derivatives; sulfated chitosan; tri or tetra-sulfated β-chitosan

Introduction

Chitin [poly(1→4)-2-acetamido-2-deoxy-β-D-glucopyranose][1] is one of the important biomass resources, but the physicochemical and biological characteristics have not been fully disclosed yet owing to the intractable nature. α-chitin has been studied most extensively because of the abundance and easy accessibility. Although only little attention has been paid to *β-chitin.* It may be a promising alternative source of chitin with distinctive features. β-Chitin is characterized by weak intermolecular forces[2] and has been confirmed to exhibit higher reactivity in various modification reactions as well as higher affinity for solvent than α-chitin.[3] It is noteworthy that β-chitosan prepared from β-chitin also exhibited high reactivity

 DOI: 10.1002/masy.200451206

compared to that from α-chitin and significant bactericidal activity.[4-7] These results suggest the high potential of chitosan derived from β-chitin as a novel functional biopolymer.

β-Chitosan is obtained by alkaline N-deacetylation of β-chitin. β-Chitosan are generally insoluble in water because of the strong intermolecular hydrogen bonding. However, they can be converting to water soluble or organic solvent soluble chitosan derivatives by chemical modification on amino, hydroxyl group of glucopyranose ring. Chitosan derivatives are generally recognized as non-toxic biomaterials with good biocompatibility. They have attracted much interest in biomedical application such as wound healing, anti microbial agents, cholesterol reducing agent, and drug delivery carrier, burn healing bio-dressing, drug delay-releasing material and blood vessel disease medicine, and food for lower blood-fat. etc. Here we report the formation of β-chitosan sulfonate derivatives prepared from our laboratory were compared with their physical property, chemical spectroscopy, ets. The degree of deacetylation (DA) and molecular weight (MW) of chitosan were determined by titration of sodium hydroxide solution and by gel permeation chromatography. These modified derivatives included sulfonate. In a current study, sulfated chitosan derivatives were synthesized to utilize chemical properties of chitosan. In addition, chitosan, as a α,β-dihydroxy propionyl[DHP] group substitution, was applied using α,β-diacetatepropionic aldehyde[DAPA]. A [DHP] contained β-chitosan derivative with large surface area -OSO_3H was then produced in order to act as heparin chemical action in solvents.

Experimental

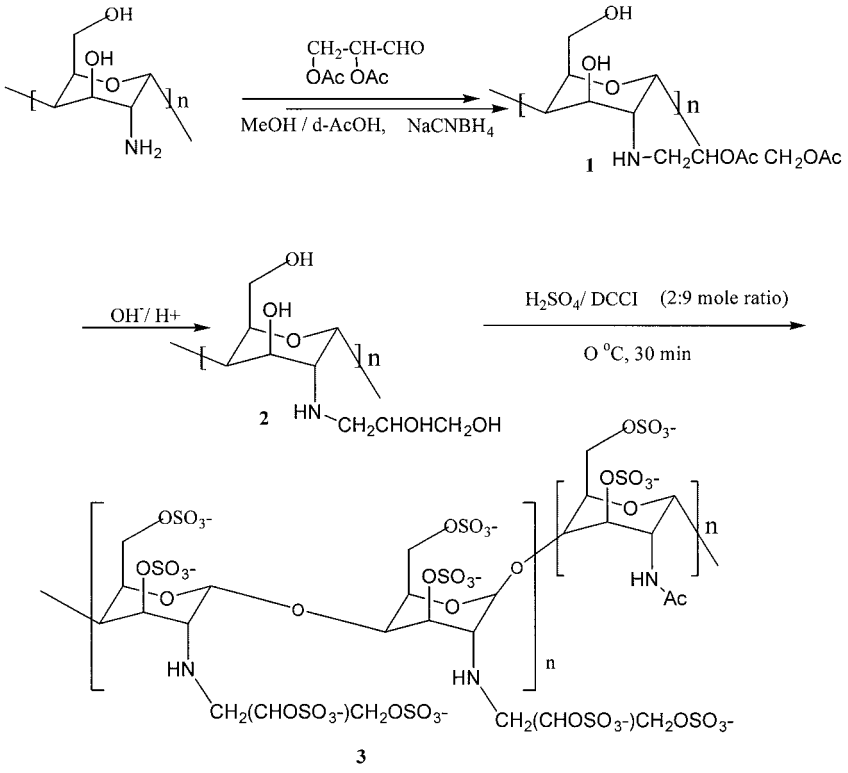
Materials

β-Chitosan was isolate from squid (*Dasidicus gigas*)pens was prepared in our laboratory as described alkali hydrolysis method. Briefly, β-chitin were treated with 40% NaOH at 100 °C for 2 h. filtered, and washed with water. The deacetylation procedure was repeated three more times to give β-chitosan. Other chemicals : acrolein, potassium permanganate, and NN'-dicyclohexylcarbodiimide(DCCI) were purchased from Wako Chemical Co., Japan. Characterization of structural changes in β-chitosan and its derivatives were determined by the Nicolet 5DX FT-IR spectrophotometer and ^{1}H NMR spectra were recorded on a Varain T60 and HA-100 spectrophotometer.

Preparation of N-(2,3-dihydroxy)propyl Chitosan Derivative (2)

β-Chitosan powder (MW 143K) 1.0 g was dissolved in 50 ml 2% (w/v) acetic acid solution,

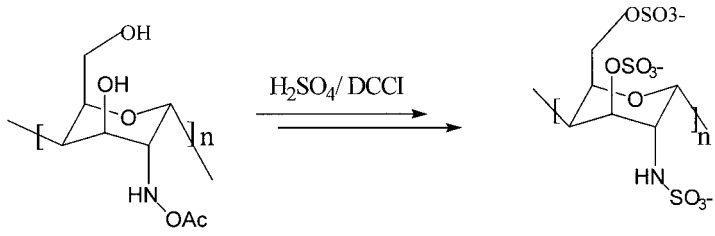
followed by adding 50 ml MeOH to prepared *dihydroxy)propyl-β*-chitosan solution. 3.0 g of 2,3-diacetylpropionicaldehyde dissolved in 30 ml methanol were added dropwise to the *β*-chitosan solution. After stirring for 12hrs at 25 ℃, the mixed solution was precipitated with 300 ml of MeOH and stirred overnight. Finally, the product was centrifuged, dialyzed with water and dried by lyophillization. and this *β*-chitin derivatives were treated with 40% NaOH at 100 °C for 2 h. filtered the solution was precipitated by adding 500 ml MeOH, followed by centrifugation and was dialyzed with distilled water. The dried product was obtained by $CaCl_2$ contained vacuum oven.



Scheme 1. Reaction pathway for synthesis of chitosan derivatives containing o-sulfate anion.

IR (KBr) spectrum shows λ_{max} (Cm^{-1}) at : 3443-3200(OH), 3240(NH), 2983, 2856(CH), 1450-1420(CH_2), 1368, 1062.

^{1}H-NMR (20% DCl/D_2O): 1.46(d, 2H, -CH_2-), 1.84(t, 1H, =CH-), 2.7(d, 2H, -CH2-), 3.68(d, 2H, -CH_2-), 3.8-4.1 (H2, H3, H4, H5, H6)



Scheme 2. Reaction pathway for synthesis of chitin derivatives containing o-sulfate anion.

Sulfonation of β-Chitosan Derivatives (SC)

β-Chitosan derivatives(2) (MW 183K) 1.5 g was dissolved in 50 ml of DMF: 2 ml of acetic acid solution to prepare β-chitosan solution10.0 ml with sulfuric acid and NN'-dicyclohexylcarbodiimide(DCCI) was added dropwise to the above viscous β-chitosan solution at 0℃, and stirred for 30 mins. The mixed solution was then stirring until to 25 ℃ and the more stirred for 6 h. The solution was diluted with 100 ml RO water and neutralized by adding 20% NaOH(aq). After neutralization, the solution was precipitated by adding 500 ml MeOH, followed by centrifugation and was dialyzed with RO water. The dried product was obtained by $CaCl_2$ contained vacuum oven.

Finally, the product was centrifuged, and filtering and It's solid gel product was hydrolysis by 20% NaOH 30 ml was added at 80 ℃ and stirred for 40min. After stirring for 30 min at 25℃. and neutralization the solution was precipitated by adding 10% HCl solution and 800 ml of MeOH, followed by centrifugation and was dialyzed with H_2O.

Sulfonation Procedure of β-chitin Derivatives

2,3-dihydroxyl-β-Chitin powder (183.2K) 1.5 g was dissolved in 60 ml DMF: 2ml acetic acid solution to prepare tetrasulfonyl-β-chitosan solution. 10.0ml with sulfuric acid and NN'-dicyclohexylcarbodiimide(DCCI) was added dropwise to the above viscous chitosan solution at 0℃, and stirred for 40 mins. The mixed solution was then warmed up to 25 ℃ and stirred for another 12 h. The solution was diluted with 100 ml RO water and neutralized by adding 10% NaOH(aq). After neutralization the solution was precipitated by adding 500 ml MeOH, followed by centrifugation and was dialyzed with RO water. The dried product was obtained by lyophilzation. α,β-dihydroxy - β-Chitosan. Molecular weight of chitosan derivatives were determined by GPC HITACHI L-7110, equipped with TSKGEL G3000PW column. The

detection was carried out with a HITACHI RI L-7490 detector. The elution was carried out with 0.2M CH_3COOH and 0.3M CH_3COONa buffer at pH 4.85. Calibration of column was carried out by the use of Shodex Standard P-82 kit with molecular weight between 800 and 5.8 kDa.

Antibacterial Test

Psedomonas areuginosa ATCC 25923 and *Staphylococcus aureus* ATCC 9027 were purchased from the Culture Collection and Research Center (Jeil pharmaceutical, Korea). All these bacteria were inoculated in 100 ml nutrient broth (NB, Merck) and incubated all 37 °C for 19 h. Various deacetylated chitosaneous derivatives and chitosan were added into 200 ml volume flask containg 100 ml KH_2PO_4 ($6.25X10^{-4}$ mol/L)buffer. 1 ml (about 10^9 CFU/ml) of each bacterium to be tested was inoculated in the flasks. Incubation was performed by shaking flask at 150 rpm, 37 °C for 1 h. 0.1 ml of decimal dilutions of samples were spared on nutrient agar plates (*Staphylococcus aureus*) and cysine repiticase agar (*Psedomonas areuginosa*) for colony counting. The inhibitory effect was calculated according to Rito -Munoz and Davidson as follows:

% inhibition= (1-T/C) 100%, C=log CFU/ml of control and T= log CFU/mol of sample

The Water Solubility (WS) of Sulfated-chitosan was measured by methods such as Ryu, etc. To begin with, three sheets of film for measuring water solubility were taken. Then, they were desiccated in the desiccators of 105°C for 24 h to measure the initial content of dry sample. Three sheets of film for measuring water solubility were separately taken and put into the beaker of 50 ml with distilled water of 30 ml. Sealing up the entrance of the beaker by parafilm, we put the beaker in the machine of normal temperature at 25°C. It was agitated sometimes and stored for 24 h. After 24 h, the film which was not resolved in the water was taken out to be desiccated by desiccators for 24 h to measure the content of dry sample. The water solubility of film was indicated by the percentage of quantity resolved in the water against the initial dry sample. The water solubility of each film was measured in 3 repetitious experiments to get average value.

Results and Discussion

The IR spectrum of N-(2,3-disulfonatepropyl)chitosan showed the presence of a carbonyl (1738 Cm^{-1}), SO_3- asymmetric stretching (1267 (Cm^{-1}), and O=S=O symmetric stretching multi peak (1027 Cm^{-1}). And, The IR spectrum of compound (3) showed the presence of

(Cm^{-1}) at: 3443-3200(OH), 3240(NH), 2983-2856(CH), 1450-1420(CH_2), 1368, 1062. The ^{1}H-NMR Spectrum also showed the existence of compound(2) δ: 1.46(d, 2H, -CH_2-), 1.84(t, 1H, =CH-), 2.7(d, 2H, -CH_2-), 3.68(d, 2H, -CH_2-), 3.8-4.1 (H2, H3, H4, H5, H6)

It was noticed that solubility of SMAC, and SC increased significantly as compared with that of unmodified β-chitosan. SMAC showed excellent inhibitory result for *Staphylococcus aeruginosa and Pseudomonas aeruginosa* than that of chitosan. In addition, SMAC showed better inhibitory data for *Pseudomonas aeruginosa* than that of SC. due to sulfur content of sulfonation. The above antimicrobial data suggested that substation ratio of sulfonation seemed to play importance role in microbial inhibition toward *Staphylococcus aeruginosa* and *Pseudomonas aeruginosa.*

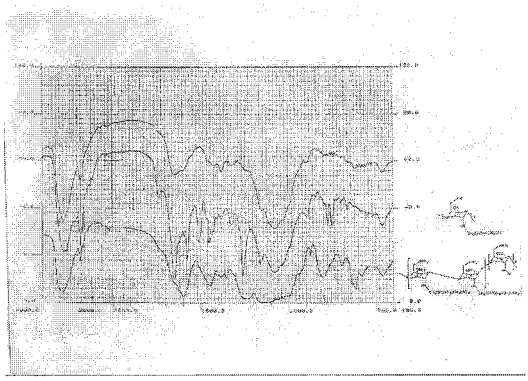


Fig. 1. IR spectrum of tetri-sulfonate polymer chitosan.

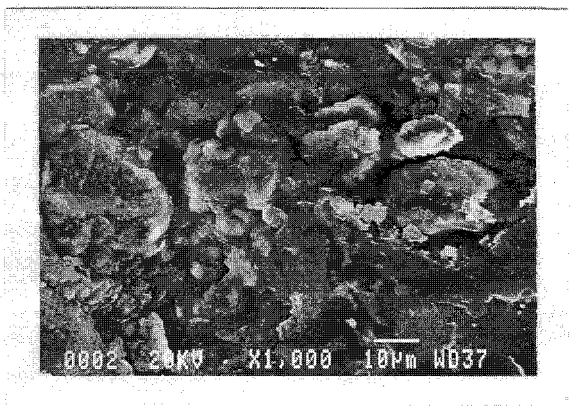


Fig. 2. SEM photograph of sulfonate β-chitosan derivative; surface(X1,000).

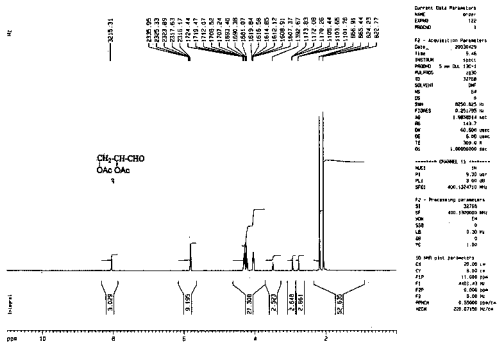


Fig. 3. IR spectrum of 2,3-diacetatylpropionic aldehyde.

Table 1. Antimicrobial Test on Chitosan Derivatives

ATCC is an abbreviation for American Type Culture Collection.

Control		β -Chitosan	SC	SMAC
Staphylococcus aureus ATCC 25923	Inhibitory	64.3	8.32	-
(%)	pH	7.5	7.8	7.5 -
Pseudomonas aeruginosa ATCC 9027	Inhibitor	83	7.1	94
(%)	pH	7.5	8.1	7.4
Escherichia coli ATCC 10536	Inhibitor	93	89	87
(%)	pH	7.5	7.9	7.43

% inhibition= (1-T/C) 100%, C=log CFU/ml of control and T= log CFU/mol of sample

Conclusions

In order to check the appropriateness for skin and pharmacological efficacy, chitosan was converted into sulfate. To be used as biological adhesive, α, β-dihydroxypropionicaldehyde was converted into sulfate by synthesizing 2 with acrolein and the following result came out.

(1) Increment of water solubility. (2) In the reaction of sulfate, the reaction of exothermic sulfuric acid was desirable and the color varied considerably due to the decomposition by temperature.

(3) For H_2SO_4: DCCI: CTS= 2: 10: 1(mole ratio), the temperature under 5°C was desirable.

(4) It seems necessary to check the behavior of electromagnetic wave scattering or gelatin in order to obtain the physical constant that is minutely comparable.

[1] K. Kurita, M. Kanari, Y. Koyama, *Polym.* **1985**, *14*: 511.
[2] J. Rudall, J. Thampson, L. Nagy, *J, Vary. Lipids* **1983**, *18*, 714.
[3] K. Kurita, K. Tomita, *Polym. Bull.* **1993**, *30*, 429
[4] M. Shimojoh, K. Masaki, K. Kurita, *Nippon Nogeikagaku Kaishi* **1996**, *70*, 787.
[5] S. ToKura, S. Baba, Y. Uraki, Y. Miura, N. Nishi, O. Hasegawa, *Carbohydrate Polymers* **1990**. *13*, 273.
[6] S. Hirano, *Carbohydrate. Res* **1976**, *47*, 315.
[7] M. Shimojo, K. Masaki, K. Kurita, K. Fukushime, *Nippon Nogeikagaku Kaishi* **1996**, *70*, 787.