Synthesis and Properties Improve of Azole Chitosan Derivatives Having a Phosphate Glucose Unit Functional Groups as an Antifungal Effect

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Chitosan is widely found in nature as shell of crab, shrimp and outer skeleton of insects and etc. It is widely used in agricultural and food-industries, cosmetics and medical fields. Special emphasis has been put on the chemical modification of chitosan to explore its full potential. The poor aqueous solubility of ketoconazole compounds[5] hinders both pharmacological studies and pharmaceutical development. To make chitosan derivatives with improved antifungal effects and solubility, some attempts have been taken for consist of antifungal group like azole derivatives onto phosphated-chitosan. The synthesized phosphate esters of azole-chitosan increased the aqueous solubility of azole-chitosan, showed high stability against chemical hydrolysis in buffer solution, and were rapidly converted to the parent drug *via* hydrolysis. phosphated-chitosan was modified with A-aldehyde using the schiff-base between the aldehyde group of *cis*-[2-(2,4-dichlorophenyl)-2-(1*H*-imidazole-yl methyl)-1,3-dioxolane-4-yl] methyl] substituted of hydroxy-3-methoxybenzaldehyde and amino group of phosphated-chitosan as reactive site. The schiff-base intermediate was then reduced with $NaBH_3CN$ to give phosphated chitosan-azole derivative form. The resulting substituted chitin and chitosan was characterized by solubility in various solvents, with enable facile characterized by IR, ^{13}C-NMR. These products were investigated for physical properties and bioactivities.

Key words: *p-chitosan, ketoconazole derivatives, polymer antifungal agents*

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. And a chemical structure of chitosan, (CTS), (poly-β-(1→4)-2-amino-2-deoxy-D-glucose) is obtained through partial deactylation of chitin, Recently CTS has attracted great attention since the range of its application has many expanded to medical, bioactivities [2-4]. drug delivery systems, wound dressing, artificial membranes among other uses [5-7] There after, the modification of its structure appears to be interesting in other to expand the potential uses of this biopolymer.

Although chitosan is structurally similar to cellulose, it has poor solubility and is more resistant to chemical reagents because of its strong micelle structure due to intermolecular or intramolecular hydrogen bonds among the hydroxyl and acetamide groups. The poor solubility of chitosan and its derivatives in common solvents has been a major drawback to its utilization.

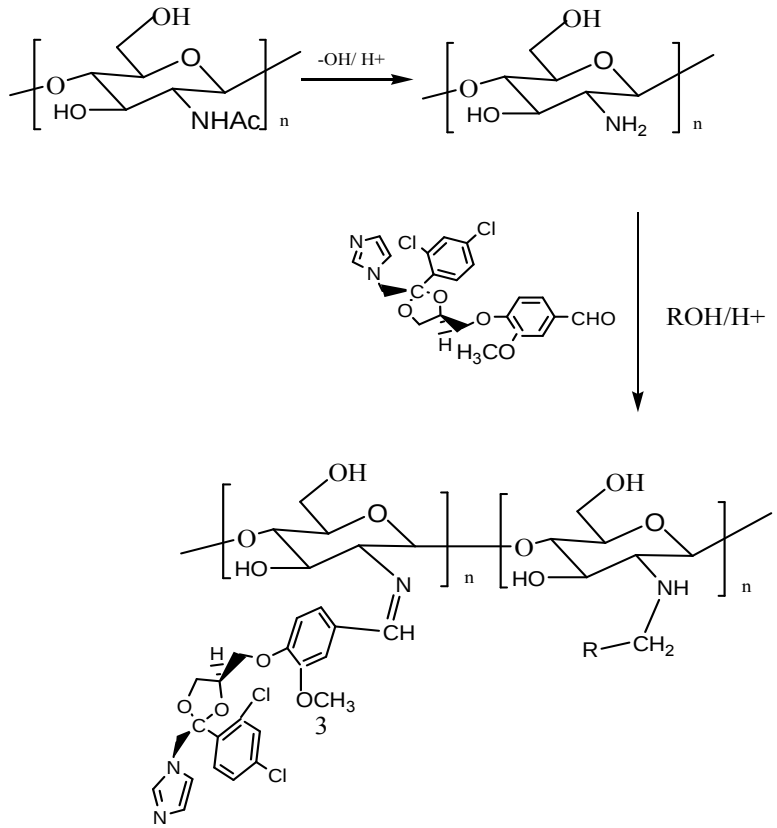
We have prepared chemically modified chitosan derivatives, which possess good solubility in many kinds of solvents and characteristic functions, in order to promote the usefulness of this polysaccharide

resource. In the course of this study, the shiff base forming in dilute acetic acid with mixed of methanol were used to be very efficient, and many kinds of alkyl-chitosans soluble in acidic solvents were successfully prepared by this method.

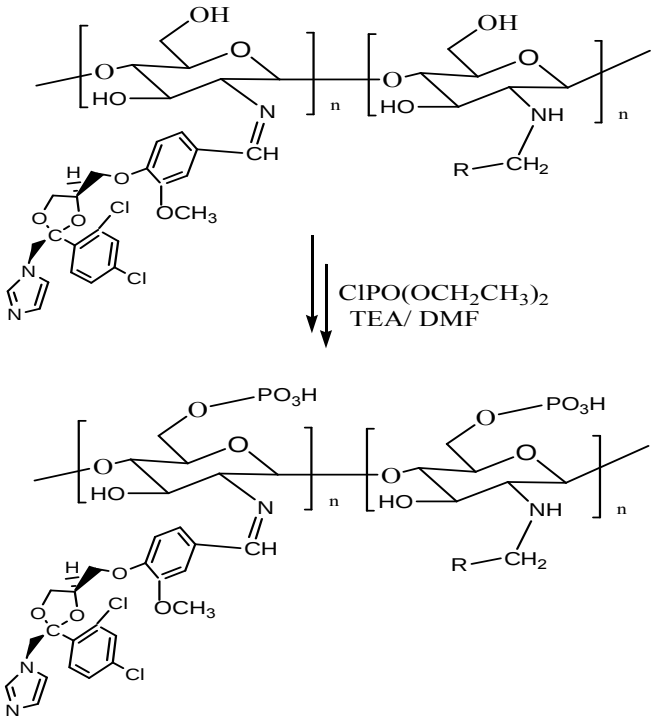
The other hand ketoconazole antifungal agents [8] bearing a 1,3-dioxolane ring at skeletal structure and a azole ring at C-2, known as azole system, are among the most important antifungal agent, showing both a high potency and a broad antifungal spectrum. In this paper we report the synthesis and the characterization of ketoconazole derivative coupled-CTS-NH_2. To make chitosan derivatives with improved hydrophilic and antifungal characteristics, some attempts have been taken for 1,3-diazole derivatives coupled polysaccaride like azolic aldehyde onto chitosan-NH_2 while keeping the fundamental skeleton of chitosan more or less intact. Azoles derivative coupled to chitosan is an antifungal agent widely used as pharmacological drugs.

Recently, the reaction of chitosan with phosphorus pentoxide by this method was found to give water-soluble phosphoric chitosan (P-chitosan) of sufficiently high degree of substitution (DS). The brief preparative procedures have been reported in a preliminary communication. This method can be applied also to the phosphorylation of variously deacetylated chitins including chitosan. Another method to prepare the P-chitin maybe possible under heating chitosan with dietoxyphosphoryl chloride and base in aprotic polar solvents by improving the procedures for phosphoric polysaccharide. Present novel procedures made it possible to produce P-chitosan of any DS, including the products of very high DS, easily and efficiently as polar property of new functional polymers.

In the present study, we aimed to prepare ketoconazole derivative coupled to water-soluble chitosan and it's phosphate chitosan derivatives. And investigate their activity behavior at various concentrations. The different states of phosphate chitosane derivatives were also determined using spectrum.



Scheme 1. Synthetic pathway of the schiff based of chitosan and ketoconazole derivatives to K-CTS



Scheme 2.. Reaction scheme for synthesis of antifungal P-K-CTS

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 phosphate. In a current study, phosphate chitosan derivatives were synthesized to utilize chemical properties of chitosan. In addition, chitosan derivative with large surface area $-OPO_3H$ was then produced in order to act as heparin ($-OSO_3H$) chemical action in a solvents.

EXPERIMENTAL

Materials

Chitosan was isolate from crap was prepared in our laboratory as previously described method [9]. Briefly, 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 chtosan. Other chemicals were purchased from Wako Chemical Co., Japan. Characterization of structural changes in chitosan and its derivatives were determined by the Nicolet 5DX FT-IR spectrophotometer and NMR experimental condition (at MNUCL): Spectrometer; Bruker Avance 400 NMR spectrometer. Solid-state nmr Prove : 4mm Double resonance MAS probe. Rotor : 4mm (o.d.) Zirconia with Kel-F cap. MAS rate : 13000 Hz for samples 2,3,4, and 5000 Hz.

Preparation of keto-chitosan (K-C)

K-C was synthesized according to the procedure reported by) J. Harris method [8]. As shown in Scheme 1, the schiff bases of K-CTS were synthesized as follows (scheme 1). keto-CHO (40 v/w) and 3g of chitosan were dissolved in 60ml of 2% acetic acid and MeOH were added with stirring at 25□. The reaction was filter mixture after stirring and washed with methanol and acetone. The product was filtrated after 30 min. and the unreacted azolic aldehydes and the other inorganic products were wash by aq-methanol and ether for 5 hrs. And it was dialyzed against deionized water for 24 h. and filtering to remove water. Swollen polymeric schiff base K-chitosan gels were dried at room temperature for 3 day, and these samples were further dried in a vacuum oven for 1 day at 50□. Antifungal assays were performed according the earlier method [9].

Preparation of phosphate keto-chitosan (P-K-C)

P-K-C was synthesized according to the procedure by use of diethoxyphosphoryl chloride (DEPC). As shown in Scheme 1, keto-chitosan and DEPC were added to DMF: benzene (1:1) solution. The

reaction mixture was stirred at 40□. After stirring and washed with methanol and acetone. This swollen polymeric P-keto chitosan gels were hydrolysis by alkali condition at 85 □ for 3 hrs and The product was filtering and it was dialyzed against deionized water for 24 h. and filtering to remove water. . Swollen polymeric chitosan gels were dried at room temperature for 3 day, and these samples were further dried in a vacuum oven for 1 day at 50□.

1.Antifungal Test

Effects of CTS-keto-P on growth of *Candida albicans*

Fungus: *Candida albicans* NIH A207 strain was used in this assay.

Anti fungal activity: *C. albicans* (1 x 10^5 cells/ml YPD*) was mixed with 1 mg/ml of samples (CTS-keto-P, and these were incubated for 24 h at 27 °C. After the incubation, the culture medium was photographed with an IX51 microscope (Olympus, Japan).

* YPD; Yeast peptone dextrose medium. It is used for cultivation of fungus.

Sustained release of antifungal agent from sample:

C. albicans (1 x 10^5 cells/ml) was planted on YPD agar, and samples (CTS-keto-P, were put on the center of YPD agar. Twenty-four hours after the incubation, clear circle by growth inhibition of *C. albicans* was observed.

Detection of antifungal agent released by samples:

CTS-keto-P was suspended in water and shaken for 5 hours. Then, the supernatant was collected and used as assay (this supernatant was called as "Sup 0-5 h"). The remained CTS-keto-P was re-suspended in water and shaken for 19 hours. Then, the supernatant was collected and used as assay (this supernatant was called as "Sup 5-24 h"). The remained CTS-keto-P was re-suspended in water and shaken for 24 hours. Then, the supernatant was collected and used as assay (this supernatant was called as "Sup 24-48 h"). To make a positive control, CTS-keto-P was suspended in water and shaken for 48 hours. The supernatant was collected and used as assay (this supernatant was called as "Sup 0-48 h"). These supernatant samples (0.01 ml) were mixed with 0.09 ml of *C. albicans* (1 x 10^5 cells/ml YPD) suspension. Twenty-four hours after the incubation, the amount of *C. albicans* cells was measured as OD6 at 20nm.

RESULT AND DISCUSSION

Poly (N-glucosamine-keto) derivative [PNGk]

Synthetic poly (N-glucosamine-keto) derivative [PNGK] was composed of chitosan and Keto-CHO group were coupled by schiff base in the presence of 2% AcOH and methanol

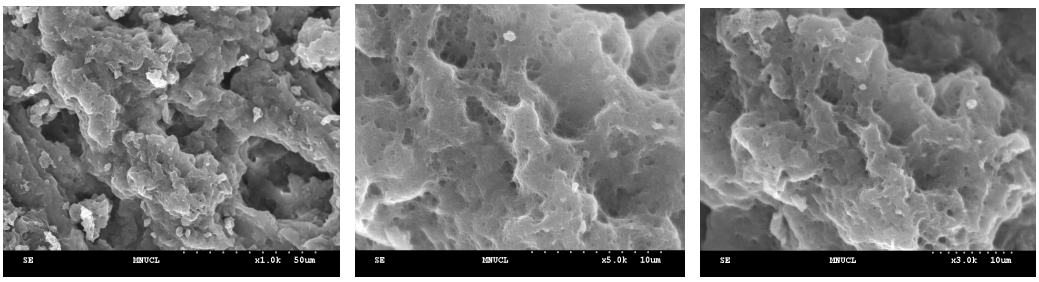


Fig 1. SEM photograph of poly (N-glucosamine-keto) derivative (X 300, 1000, 3000).

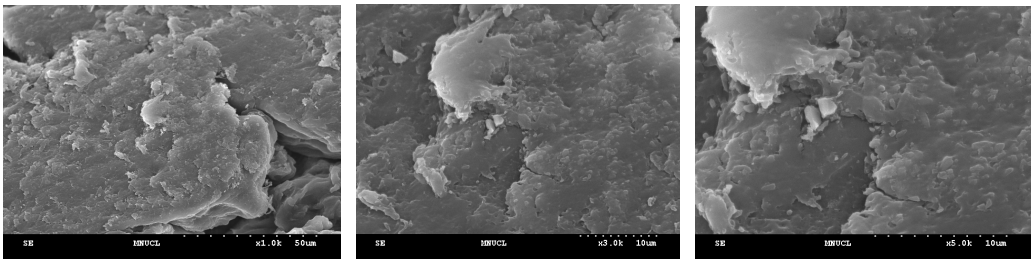


Fig 2. SEM photograph of P-K-CTS derivative (X 300, 1000, 3000)

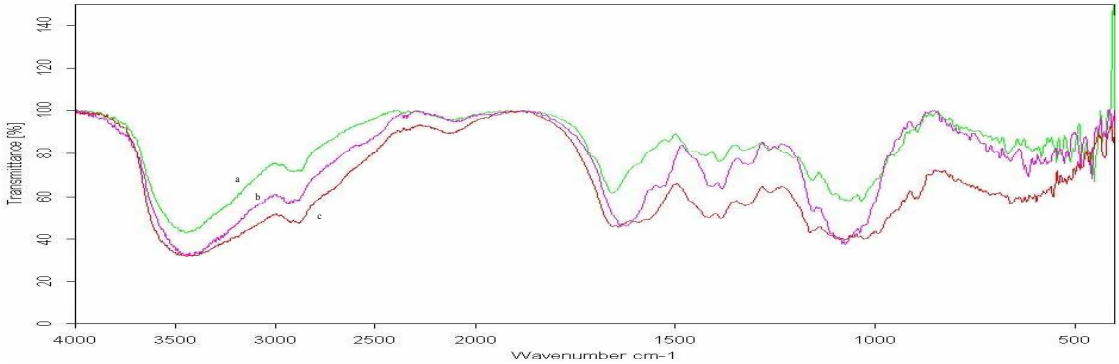


Fig 3. IR spectrum of CTS (a), K-C (b), P-K-C (c) derivatives.

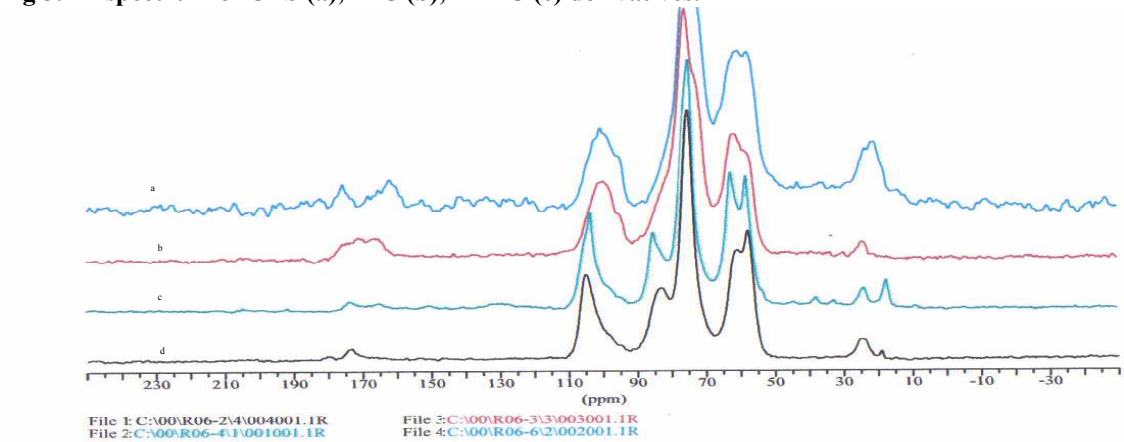


Fig 4. ^{13}C-SNMR Spectra of CTS(c, d), P-Keto-CTSate (a), P-Keto-CTS (b).

The synthesis pathway of keto-chitosan derivatives, the azole antifungal agent modified the ways that Heer's etc. have been reported, synthesized ketal that is a basic structure of azole compounds by reaction of 1,3-dichloroacetophenone and glycerin, and then synthesized a azole compound containing a new 1,3-dioxolane structure forming the benzaldehyde derivative compound. For the synthesis method in which coupled azoles from intermediate compound by using each constituents compound, we synthesized poly (N-glucosamine-keto) derivative P-Keto-CTS, the new antifungal compounds.

1. Results

Anti fungal activity:

To confirm the anti-fungal activities of CTS-keto-P, these samples were mixed with *C. albicans*. Twenty-four hours after the incubation, the culture medium was observed (Fig. 1). The cultured medium added with CTS-keto-P was cleared, because *C. albicans* could not grow in this condition.

Sustained release of antifungal agent from sample:

To confirm whether a soluble antifungal agent was released from these samples, the effects of samples (CTS-keto-P) on growth of *C. albicans* on YPD agar were monitored. CTS-keto-P is low soluble material, however, the growth of *C. albicans* around this sample was inhibited (Fig. 2). This result indicated that soluble antifungal agent was released concentrically from CTS-keto-P

Detection of antifungal agent released by samples:

To monitor the sustainable time of antifungal agent in CTS-keto-P, the antifungal activity in supernatant of CTS-keto-P suspension was measured. As shown in Fig. 3, antifungal activity was detected in sup 24-48. This result indicated that CTS-keto-P can sustained antifungal agent more than 24 hours.

We concluded that CTS-keto-P may be useful for sustained release drug.

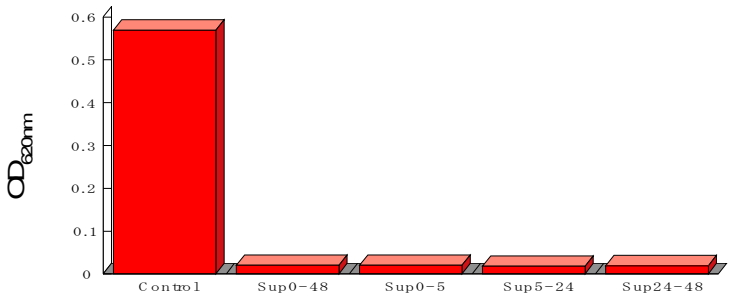


Fig. 3
Detection of antifungal agent released by samples

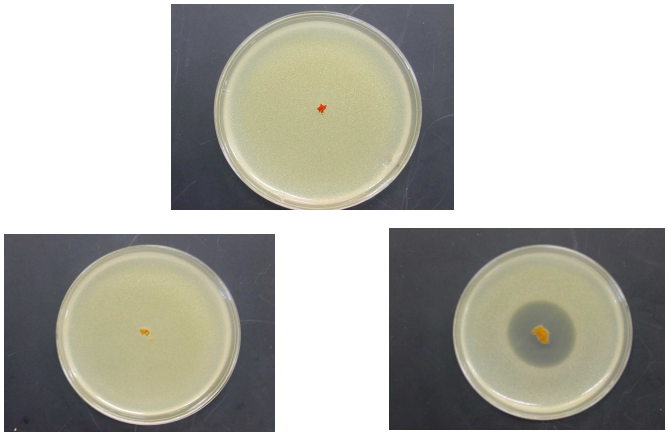


Fig. 2
Release of anti-*Candida* agent from CTS-keto-P

CONC

Micro edicine Tech Co. were synthesized with ketoconazole derivative, a well-known antifungal agent. To improve the chemical compound's solubility and cellular permeability, phosphorous acid was mixed with carbohydrate. The study on this compound's minimum growth suppression concentration and inhabitation found the following conclusions:

1. The MIC value of Low and P-Poly with different chitosan molecular weights was 0.019±0.003 for the ketoconazole derivative with the molecular weight of 13KDa and 0.014±0.005 for the ketozonazole derivative with the molecular weight of 36Kda for 0-2hr elution. In result, these samples showed similar antifungal activity compared to chitosan derivative with higher molecular weight (36KDa).
2. Chitosan derivative's MIC value for inhibited the growth of *C. albicans*, activity result, was 0.01-0.05% and its antifungal activity was very high compared to its MIC control value Low:0.088±0.012. P-Poly: 0.088±0.012 for 0-4hr elution.
3. The detailed structure of bacteria influenced by chitosan was observed under a microscope to examine the antifungal activity of chitosan derivative and it was found that cell wall and cell

membranes had big injuries and interfered with the life activities of chitosan processing microorganisms.

4. As chitosan molecular weight affects its antifungal activities, chitosan derivative containing compounds with outstanding antifungal activity and appropriate molecular weight would be considerably helpful to develop naturally high-molecular and anti-microorganism chitosan. In addition, the new development would be suitable for various industries and medicines.

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