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Preparation and antimicrobial activity of hydroxypropyl chitosan

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Abstract—Water-soluble hydroxypropyl chitosan (HPCS) derivatives with different degrees of substitution (DS) and weight-average molecular weight (M_w) were synthesized from chitosan and propylene epoxide under basic conditions. Their structure was characterized by IR spectroscopy, NMR spectroscopy, and elemental analysis, which showed that both the OH groups at C-6 and C-3 and the NH_2 group of chitosan were alkylated. The DS value of HPCS ranged from 1.5 to 3.1 and the M_w was between 2.1×10^4 and 9.2×10^4. In vitro antimicrobial activities of the HPCS derivatives were evaluated by the Kirby–Bauer disc diffusion method and the macrotube dilution broth method. The HPCS derivatives exhibited no inhibitory effect on two bacterial strains (*Escherichia coli* and *Staphylococcus aureus*); however, some inhibitory effect was found against four of the six pathogenic fruit fungi investigated. Some derivatives (HPCS1, HPCS2, HPCS3, HPCS3-1, and HPCS4) were effective against *C. diplodiella* and *F. oxysporum*. HPCS3-1 is the most effective one with MIC values of 5.0, 0.31, 0.31, and 0.16 mg/mL against *A. mali*, *C. diplodiella*, *F. oxysporum*, and *P. piricola*, respectively. Antifungal effects were also observed for HPCS2 and HPCS3-1 against *A. mali*, as well as HPCS3 and HPCS3-1 against *P. piricola*. The results suggest that relatively lower DS and higher M_w value enhances the antifungal activity of HPCS derivatives.

Keywords: Hydroxypropyl chitosan; Antifungal activity; *Altwenaria mali*; *Coniella diplodiella*; *Fusarium oxysporum*; *Physaclospora piricola*

1. Introduction

As a natural renewable resource, chitosan has a number of unique properties such as biocompatibility, biodegradability, non-toxicity, and antimicrobial activity, which have attracted much scientific and industrial interest in such fields as biotechnology, pharmaceutics, wastewater treatment, cosmetics, agriculture, food science, and textiles.[1] Although chitosan is soluble in aqueous dilute acids below pH 6.5, it is insoluble in water and most organic solvents. The poor solubility of chitosan is a major limiting factor to its utilization. Therefore, special attention has been paid to its chemical modification and depolymerization to obtain derivatives soluble in water over a wider pH range.[2]

Interestingly, many new uses have been found in the derivatives and oligomers of chitosan apart from their enhanced water-solubility.[3] For example, phosphorylated chitosan exhibits a significant anti-inflammatory effect against hemorrhagic pneumonia[4] and amino-derivatized cationic chitosans elicit dose-dependent inhibitory effects on proliferation of tumor cell lines.[5] Chitosan sulfates also have anticoagulant activity, and carboxymethyl chitosan sulfate inhibits the transformation of fibrinogen to fibrin due to its structural similarity to heparin.[6,7] Furthermore, antimicrobial activities of chitosan derivatives have received considerable attention in recent years due to problems associated with chemical fungicide agents.[8] It has been reported that quaternary ammonium salts of chitosan exhibited good antibacterial activities[9] and a quaternized diethylmethyl chitosan chloride showed higher antibacterial activity than chitosan.[10] In addition, novel *N*,*O*-acyl chitosan derivatives were more active against the gray mold

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fungus *Botrytis cinerea* and the rice leaf blast fungus *Pyricularia oryzae* than chitosan itself.[11]

Hydroxypropyl chitosan (HPCS) is another important functional derivative of chitosan. Liquid crystal phases, foam performance, and emulsifying power have been observed in solutions of HPCS.[12,13] Wang found that HPCS has a high anticoagulant effect, and the clinical effect of artificial tears based on HPCS was better than hydroxypropylmethylcellulose on dry eye disease.[14,15] In addition, HPCS as a reaction intermediate can be further modified. HPCS grafted with maleic acid were found to kill 99.9% of *Staphylococcus aureus* and *Escherichia coli* within 30 min at the concentration of 100 ng/mL.[16] However, there are few reports on the antimicrobial activities of HPCS. The present work is aimed at the preparation of a series of HPCS derivatives with different molecular weight and degree of substitution, and the investigation of their antimicrobial activities.

2. Experimental

2.1. Materials

Five chitosan samples designated as CS1, CS2, CS3, CS4, and CS5 from crab shells were supplied by Haili Biologic Products Co. Ltd. (China), and re-purified by washing in succession with 1% NaOH aqueous solution, distilled water, and alcohol. Another two chitosan samples, designated as CS3-1 and CS3-2, were obtained through ultrasonic degradation as follows: CS3 was dissolved in 1% acetic acid and then ultrasonically degraded (KQ-250DB, China) at an energy level of 150 W at 80 °C for 2 and 8 h, respectively. The degraded solution was neutralized with 0.1 M NaOH, and the precipitate was collected, washed with distilled water and alcohol, and vacuum dried. The degree of deacetylation (DD) (Table 1) was determined by titration.[17]

An oligochitosan (M_w = 2500, DD = 93%) was also purchased from the Haili Biologic Products Co. Ltd. (China) and used without further purification. Propylene epoxide was of chemical grade and purchased from Shanghai Medicine Group (China). All other solvents and reagents were used as received. Two bacteria, *E. coli* and *S. aureus*, were supplied by the Laboratory of Microbiology, Ocean University of China. Six fruit pathogenic fungi, *Coniella diplodiella*, *Rhizopus nigricans*, *Gloeosporium fructigenum*, *Fusarium oxysporum* sp., *Altwenaria mali*, and *Physaclospora piricola*, were obtained from the Qingdao Academy of Agriculture Sciences, China.

2.2. Preparation of hydroxypropyl chitosan

The seven chitosan samples (CS1, CS2, CS3, CS3-1, CS3-2, CS4, and CS5) were hydroxypropyl-etherified individually according to the literature[16] to give the corresponding hydroxypropyl chitosan derivatives encoded as HPCS1, HPCS2, HPCS3, HPCS3-1, HPCS3-2, HPCS4, and HPCS5. In brief, 5.0 g chitosan was added to 50 mL 33% NaOH aqueous solution, stirred for 2 h at room temperature and then kept for 10 days at −18 °C. The mixture was then thawed and filtered through sand filter to provide basified chitosan, which was transferred to a flask containing 150 mL isopropyl alcohol. After vigorously stirring for an hour, 75–100 mL propylene oxide was added drop-wise with stirring over 1 h. The suspension was further stirred at 45 °C for 8–16 h. The resulting precipitate was neutralized by the addition of hydrochloric acid, and then dialyzed using a regenerated cellulose tube (M_w cut-off 8000, Union Carbide, USA) against distilled water for 3 days. The resulting solution was subsequently concentrated by rotary evaporation at reduced pressure below 60 °C and lyophilized to give the colorless hydroxypropyl chitosan (HPCS) derivatives.

2.3. Characterization of HPCS derivatives

The ^{1}H NMR spectrum was recorded on an INOVA-600 spectrometer (Varian Inc., America) at 600 MHz at 25 °C, using D_2O as the solvent. CHN elemental analysis was measured by an Elemental Analyzer-MOD 1106 (Carlo Erba Strumentazione). Infrared spectra were recorded in a KBr disk using a Nicolet 170SX FT-IR (Perkin Elmer Co., USA) spectrometer equipped with DGTS detector and DMNIC 3.2 software over the range of 4000–400 cm^{-1}.

2.4. Size exclusion chromatography measurement

Size exclusion chromatography (SEC) measurement was performed on a Waters instrument combined with a binary HPLC pump equipped with TSK-GEL G4000PWXL column (7.8 mm × 300 mm) and Waters 2414 refractive index detector at 25 °C. The eluent was 0.1 mol/L NaCl aqueous solution and the flow rate used was 1.0 mL/min. All HPCS samples were first dissolved in 0.1 mol/L NaCl aqueous solution at a concentration

Table 1. Degree of deacetylation (DD) and M_w of chitosan samples

Sample	CS1	CS2	CS3	CS3-1	CS3-2	CS4	CS5
DD (%)	91.0	91.0	90.9	90.7	91.3	91.0	91.0
$M_w \times 10^{-4}$	67.0	60.1	41.0	35.7	29.8	17.1	14.7

of 1.0 mg/mL, then filtered through sand filter and a 0.20 μm filter (Whatman, UK); 200 μL were injected in each run. The columns were pre-calibrated using pullulan (Showa Denko, Japan) with different molecular weights as the standards. The average molecular weights of HPCS were estimated based on the calibration curve of pullulan.

2.5. Viscometry

The viscosities of chitosan fractions in 0.1 mol/L CH_3COONa + 0.2 mol/L CH_3COOH solution were determined by the Ubbelohde type viscometer at 30 ± 0.1 °C. The kinetic energy correction was negligible. The Huggins and Kraemer equations were used to estimate the intrinsic viscosity [η]. The weight-average molecular weights were calculated by Mark–Houwink equation[18]

$$[\eta] = kM_w^{\alpha}$$

where $k = 1.64 \times 10^{-30} \times DD$,[14] $\alpha = -1.02 \times 10^{-2} \times DD + 1.82$, and DD is the degree of deacetylation of chitosan. The weight-average molecular mass (M_w) of HPCS samples is listed in Table 1.

The viscosities of HPCS samples in 0.1 mol/L NaCl aqueous solution were also determined by the Ubbelohde type viscometer at 25 ± 0.1 °C, which are shown in Table 3.

2.6. Evaluation of antimicrobial activity

Solutions of HPCS were prepared by dissolving powders in distilled water and sterilizing them at 115 °C for 15 min before use. Strains of *E. coli* and *S. aureus* were cultured on nutrient agar slopes (peptone 1%, NaCl 0.5%, beef extract 0.3%, agar 2%, pH 7.4). Suspensions were prepared by transferring sterile 0.9% saline to the slopes, and a final concentration of 1.0×10^6 CFU/mL were obtained as diluents for the antibacterial test. The suspensions of *C. diplodiella*, *R. nigricans*, *G. fructigenum*, *F. oxysporum*, *A. mali*, and *P. piricola* were prepared similarly on Sabouraud dextrose slopes (glucose 4%, peptone 1%, agar 2%, pH 6.0), and a final concentration of 1.0×10^5 CFU/mL was obtained by appropriately diluting the slopes with a sterile 0.9% saline, which were used for the antifungal test.

In vitro antimicrobial activities of HPCS were first determined against the test bacteria and fungi by the Kirby–Bauer disc diffusion method.[19] The assay plate was seeded with 100 μL microbial suspension, then three filter papers (diameter of 5 mm) containing a sample solution (1% in distilled water, w/v) were placed on the plate, respectively. Filter papers containing distilled water were used as control. The plates being seeded with *E. coli* and *S. aureus* were incubated at 37 °C for 24 h, those being seeded with *C. diplodiella*, *R. nigricans*, *G. fructigenum*, *F. oxysporum*, *A. mali*, and *P. piricola* were incubated at 28 °C for 48 h, after which the average diameter of the inhibition zones were measured.

For those fungi where obvious inhibition zones were found, their minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were further determined by the macrotube dilution broth method according to the National Committee for Clinical Laboratory Standards (NCCLS, 1997).[20] First, 1.0 mL sterile broth was added to each tube, followed by the addition of 1.0 mL HPCS solution to the first tube. After mixing, 1.0 mL of this solution was transferred to the second tube. The dilution process was continued to provide a total of 12 tubes; the thirteenth tube served as a control and no sample solution was added to it. Finally, 100 μL of the microbial suspension was added to each tube. The MIC was taken as the lowest concentration of HPCS at which the microorganism tested did not show visible growth after incubation at 28 °C for 72 h. From the tubes without visible fungus growth, 100 μL of broth was transferred onto Sabouraud dextrose agar and spread across the entire surface of the plate. All experiments were carried out in triplicate. The MBC was taken as the average lowest concentration at which no colony growth was found after incubation at 28 °C for another 72 h. A water-soluble oligochitosan (M_w = 2500, DD = 93%) was also examined as a control.

3. Results and discussion

3.1. Characterization of HPCS

Figure 1 shows the IR spectra of chitosan (CS3), ultrasonically degraded chitosan (CS3-1), and HPCS samples (HPCS3, HPCS3-1, and HPCS5). The IR spectrum of CS3-1 was nearly the same as that of CS3. Both

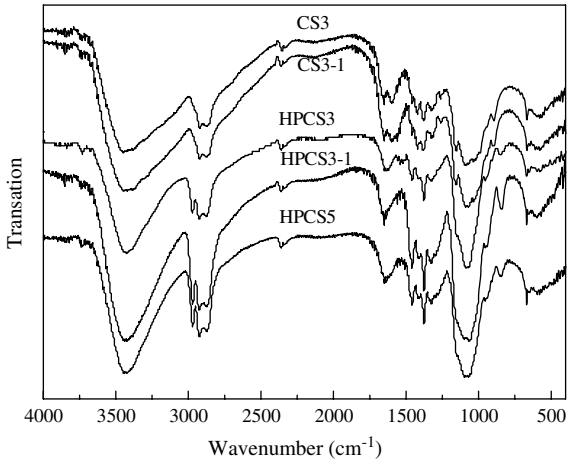

Figure 1. FTIR spectra of CS3, CS3-1, HPCS3, HPCS3-1, and HPCS5.

Table 2. Elemental analysis results of HPCS samples

Sample	N (%) Found (calculated)	C (%) Found (calculated)	H (%) Found (calculated)	DS
HPCS1	4.76 (4.74)	52.94 (52.62)	8.23 (8.43)	2.32
HPCS2	5.37 (5.41)	50.98 (51.28)	8.02 (8.16)	1.69
HPCS3	4.52 (4.55)	52.71 (53.00)	8.54 (8.51)	2.54
HPCS3-1	5.68 (5.68)	50.71 (50.73)	8.43 (8.05)	1.47
HPCS3-2	4.08 (4.09)	53.76 (53.89)	8.45 (8.69)	3.12
HPCS4	4.97 (5.01)	51.71 (52.08)	8.13 (8.32)	2.04
HPCS5	4.27 (4.30)	53.10 (53.50)	8.01 (8.61)	2.84

exhibited the absorption peaks at 1152, 1082, 1028, and 897 cm^{-1}, which could be assigned to the saccharide moiety. The peaks at 1655 and 1599 cm^{-1} could be attributed to the carbonyl stretching $\nu_{C=O}$ (amide I) and amine bending δ_{N-H} (amide II), respectively.[21] In the IR spectra of the HPCS samples, new absorption peaks appeared at 2970 and 1376 cm^{-1}, corresponding to the C–H stretching and bending of the CH_3 group. These absorptions indicate that CH_3 group was introduced into the chain of chitosan after reaction with propylene epoxide.[22] In addition, the absorption peaks at 1030 and 1160 cm^{-1}, which were attributed to ν_{C-O} of 3-OH and 6-OH of chitosan, respectively, nearly disappeared, implying that the hydroxypropyl substitution occurred at both 3-OH and 6-OH groups.[9] Moreover, the characteristic peaks at 1599 cm^{-1} were substantially weakened, representing a decrease of $-NH_2$ group content.[23] These results revealed that both the OH groups at C-6 and C-3 and the NH_2 group could be alkylated under the experimental condition.

The elemental analysis results and the degree of substitution (DS) value of HPCS samples are summarized in Table 2. DS, which was designated as the average number of hydroxypropyl groups on each sugar residue, was calculated by C%/N%. The deacetylation degrees of HPCS samples were taken as 100% due to the deacetylation that occurred during the course of alkalization and alkylation of chitosan. The obtained DS values of HPCS samples were from 1.5 to 3.1.

^{1}H NMR analysis was employed for further estimation of the DS value of the HPCS samples. Figure 2 illustrates the ^{1}H NMR spectrum of HPCS3. The DS value could be calculated by the following formula:[12]

$$I_{H9}/I_{H2-8} = 3\,DS/(6 + 3\,DS)$$

where I_{H9} and I_{H2-8} are the absorption intensity of hydrogen at C-9 and hydrogens at C-2 to C-8, respectively. The DS of HPCS3 was 2.8, which was consistent with the result from the elemental analysis. The signal at δ 2.02, corresponding to *N*-acetamido group of the parent chitosans, was essentially absent demonstrating that essentially complete deacetylation had occurred during the basification and alkylation of chitosan.

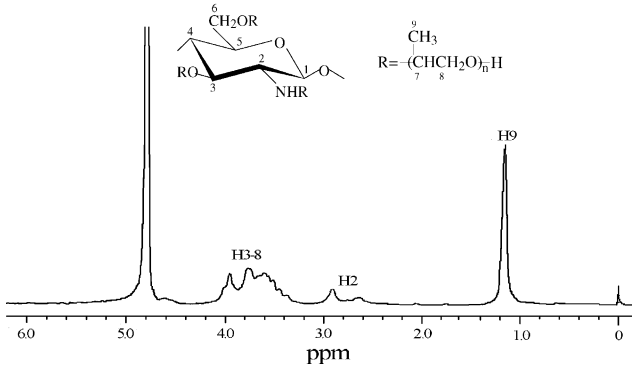

Figure 2. ^{1}H NMR spectrum of HPCS3 in D_2O at 25 °C.

The values of [η] and M_w of HPCS samples are listed in Table 3. Compared with the high M_w values of unmodified chitosan, the low M_w values of corresponding HPCS samples indicated that the chitosan chain degraded during alkalization and alkylation. However, the [η] values of HPCS samples were relatively high, which was probably because the HPCS samples were very soluble in 0.1 mol/L NaCl aqueous solution at room temperature, and thus exhibited an expanded chain conformation.[24]

3.2. Antimicrobial activities of HPCS

The capabilities of HPCS in inhibiting the growth of the tested microbes on solid media are listed in Table 4. No inhibition zone was observed for the seven HPCS samples against the two bacteria, *E. coli* and *S. aureus*. It is well known that chitosan and its derivatives presented significant bactericidal effects against both Gram-positive and Gram-negative bacteria below pH 6 due to the positively charged amino group, which can interact with negatively charged bacterial cell membranes.[25]

Table 3. Intrinsic viscosity [η] and M_w of HPCS samples in 0.1 mol/L NaCl aqueous solution at 25 °C

Sample	HPCS1	HPCS2	HPCS3	HPCS3-1	HPCS3-2	HPCS4	HPCS5
[η] (mL/g)	54.1	49.1	194	125	48.2	42.2	34.8
$M_w \times 10^{-4}$	5.6	4.2	9.2	8.0	3.2	2.2	2.1

Table 4. Diameters of inhibition zones (mm) of HPCS samples (1% in distilled water) against *A. mali*, *C. diplodiella*, *F. oxysporum*, and *P. piricola*

Sample	HPCS1	HPCS2	HPCS3	HPCS3-1	HPCS3-2	HPCS4	HPCS5
A. mali	—	7.4	—	7.5	—	—	—
C. diplodiella	9.0	8.3	8.3	8.8	10.3	9.3	12
F. oxysporum	8.0	8.8	8.1	9.0	7.5	8.3	7.5
P. piricola	9.3	10.7	8.0	8.8	—	—	—

—: No obvious inhibition zone was observed in Sabouraud dextrose agar plates.

Therefore, the poor inhibitory effect of HPCS on *E. coli* and *S. aureus* can possibly be attributed to the relatively small number of charged amino groups in the molecules. In contrast, HPCS samples showed antifungal activity to some extent. HPCS2 and HPCS3-1 inhibit growth of four out of the six fruit pathogenic fungi. The most sensitive fungi were *C. diplodiella* and *F. oxysporum*, which were inhibited by all the HPCS samples.

3.3. MICs and MBCs of HPCS against *A. mali*, *C. diplodiella*, *F. oxysporum*, and *P. piricola*

Based on the results from Table 4, the inhibitory effects of HPCS samples against *A. mali*, *C. diplodiella*, *F. oxysporum*, and *P. piricola* were further estimated by the macrotube dilution broth method. An oligochitosan was also tested as a control. Table 5 illustrates the MICs and MBCs of HPCS and oligochitosan against the four fungi. The results indicated that HPCS3-2 and HPCS5 were inactive against the four fungi tested, and their MIC values could not be determined as they are likely much higher than the concentration tested. HPCS1, HPCS2, HPCS3, HPCS3-1, and HPCS4 were all active against *C. diplodiella* and *F. oxysporum*. HPCS2, HPCS3, and HPCS3-1 were more active than HPCS1 and HPCS4, and HPCS3-1 was the most active one as indicated by the MIC (MBC) values of 0.31 and 0.31 mg/mL against *C. diplodiella* and *F. oxysporum*, respectively. HPCS3 and HPCS3-1 were effective in inhibiting the growth of *P. piricola* with MIC values of 0.55 and 0.16 mg/mL, respectively. Antifungal effects were also observed for HPCS2 and HPCS3-1 against *A. mali* with relatively high MIC values of 4.4 and 5.0 mg/mL, respectively.

Table 5. MIC and MBC values of HPCS samples and oligochitosan against *C. diplodiella*, *F. oxysporum*, *A. mali*, and *P. piricola*

Sample	MIC/MBC (mg/mL)			
	A. mali	*C. diplodiella*	*F. oxysporum*	*P. piricola*
HPCS1	—	5.0/10	10/10	—
HPCS2	4.4/8.8	0.55/1.1	0.55/4.4	
HPCS3	—	1.1/4.4	1.1/1.1	0.55/2.2
HPCS3-1	5.0/20	0.31/0.31	0.31/0.31	0.16/0.16
HPCS3-2	—	—	—	—
HPCS4	—	7.5/7.5	3.8/15	—
HPCS5	—	—	—	
Oligochitosan	1.0/2.0	0.13/0.25	2.0/4.0	1.0/2.0

—: Not determined under the tested condition.

As shown in Tables 2 and 3, the seven HPCS samples had different DS and M_w values, which indeed resulted in the differences of their antifugal activities. HPCS3-2 and HPCS5 had the highest DS of the HPCS samples, but no antifungal effect. These results imply that high DS causes loss of antifungal activity of HPCS. HPCS2 and HPCS3-1 had the lowest DS values (1.7 and 1.5, respectively), moderately high M_w, and inhibited the growth of *A. mali*. HPCS3 and HPCS3-1 had the highest M_w value and showed antifungal activity against *P. piricola*. Therefore, it is reasonable to infer that relatively lower DS and higher M_w enhances the inhibitory activity of HPCS against some fruit pathogenic fungi.

Although the exact antifungal mechanism of HPCS was still unknown, it has been reported that chitosan has dual functions: direct interference of fungal growth and activation of several defense processes.[26] The defense mechanisms included the accumulation of chitinases, synthesis of proteinase inhibitors, and induction of callous synthesis.[27]

The inhibitory effect of HPCS depended on the cell structure of the fruit pathogenic fungi. As noted previously, chitosan reduced the in vitro growth of numerous fungi with the exception *Zygomycetes*, which contained chitosan as a major component of the cell walls.[28] El Ghaouth et al. showed that the positively charged groups along the length of chitosan were important because low antifungal activity was observed with *N*,*O*-carboxymethylchitosan compared to chitosan itself.[29] In addition, *N*-dicarboxymethyl chitosan also seemed to favor the growth of *Saprolegnia parasitica*.[30] It was also found that the antibacterial activity of quaternary ammonium chitosan salts increased with increasing chain length of the alkyl substituent, and this was attributed to the increased hydrophobic properties of the derivatives.[31] Therefore, the antifungal effect of HPCS samples probably resulted from their good water-solubility and some hydrophobic property due to the introduction of hydroxypropyl group.

4. Conclusion

In summary, water-soluble hydroxypropyl chitosan (HPCS) derivatives were prepared and characterized in this study. Hydroxypropylation occurred at both the 6-OH and 3-OH groups as well as the NH_2 group of

the chitosan. The DS value of HPCS was from 1.5 to 3.1 and the M_w was between 2.1×10^4 and 9.2×10^4. HPCS exhibited no inhibitory effect on the bacteria *E. coli* or *S. aureus*. However, HPCS samples were inhibitory effective against the four fruit pathogenic fungi tested. HPCS3-1 appeared to be the most active one, with an MIC of 5.0, 0.31, 0.31, and 0.16 mg/mL to *A. mali*, *C. diplodiella*, *F. oxysporum*, and *P. piricola*, respectively. Relatively lower DS and higher M_w value of HPCS could result in higher antifungal activity.

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