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Synthesis, Characterization, and Antimicrobial Activity of Kojic Acid Grafted Chitosan Oligosaccharide

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Supporting Information

ABSTRACT: A novel water-soluble chitosan oligosaccharide (COS) derivative, chitosan oligosaccharide/kojic acid grafts assigned as COS/KA, was prepared by using the selective partial alkylation of *N*-benzylidene COS and chlorokojic acid in the presence of dimethyl sulfoxide (DMSO) and pyridine (Py). The derivative was characterized by UV−vis spectroscopy, FTIR, ^{1}H NMR, TGA, SEM, and XRD techniques, which showed that the alkylation reaction took place at the C-6 and C-3 positions of COS. The results showed that the degree of substitution (DS) for COS/KA was from 0.38 to 1.21, and the product exhibited an excellent solubility in organic solvents and distilled water. The antibacterial results indicated that the antibacterial activity of COS/KA was strengthened relative to COS with the increase of DS for *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger* and *Saccharomyces cerevisiae*. These findings provide important supports for developing new antibacterial agents and expand the scope of application of COS in the food industry.

KEYWORDS: *chitosan oligosaccharide, kojic acid, antimicrobial activity*

INTRODUCTION

Many researchers have focused on chitosan as a source of potential bioactive material during the past few decades. However, it has several drawbacks for its utilization in biological applications, including poor solubility and absorption under physiological conditions.[1] Unlike chitosan, chitosan oligosaccharide (COS), the hydrolyzed product of chitosan, is a mixture of oligomers of β-1,4-linked D-glucosamine residues that have better biocompatibility and solubility due to their shorter chain lengths and free amino groups in D-glucosamine units.[2] Many of the biological activities reported for COS, such as antifungal,[3,4] antibacterial,[5] and antitumor,[6] are dependent on their physicochemical properties, which allow COS to be considered as a potential novel functional food ingredient, particularly in the preparation of low-calorie foods.[7] Recently, it has been shown that COS and its derivatives exert antimicrobial effects against different groups of microorganisms such as bacteria, fungi, and yeast.[7] Therefore, it has also been used as an antibacterial agent and additive to improve the shelf life of food products.[8] There are three types of reactive functional groups present in COS (an amino group as well as both primary and secondary alcoholic OH groups at the C-2, C-3, and C-6 positions, respectively), which can be readily subjected to chemical derivatization, allowing its antibacterial activity to be increased.[9] However, the antibacterial activity of COS is low, and chemical modification may lead to enhancement of its antibacterial activity.

Kojic acid (KA), 2-hydroxymethyl-5-hydroxy-4*H*-pyran-4-one, which is an organic acid, is a metabolic compound produced by several species of economically valuable fungi, such as *Aspergillus*, *Acetobacter*, and *Penicillium*.[10] At present, kojic acid and its derivatives have drawn attention because they have been shown to possess various bioactivities such as antimicrobial and antiviral,[11] anti-inflammatory,[12] antitumor,[13] antidiabetic,[14] and skin-whitening activities.[15] Recently, methods for the synthesis of various kojic acid derivatives, such as kojic acid esters kojic acid laureate and kojic acid dipalmitate, have been reported in many studies.[16,17] Moreover, kojic acid provides a promising skeleton for development of new more potent derivatives such as chlorokojic acid (2-chloromethyl-5-hydroxy-4*H*-pyran-4-one), which is a good ligand for the nucleophilic and electrophilic substitution reaction depending on the reagent type and can inhibit *Aeromonas aerogenes*, *Micrococcus pyogenes* var. *aureus*, *Salmonella typhosa*, *Penicillium digitalum*, *Russula nigricans*, and *Saccharomyces cerevisiae* growth.[10] Marwaha and co-workers reported that organomercury(II) complexes of kojic acid and maltol demonstrated greater antibacterial activity than kojic acid.[18] Nevertheless, at present, there is little information available regarding the synthesis of COS/KA graft complexes and their antibacterial activity.

Both COS and kojic acid are good natural food preservatives for improving food quality and safety. Herein we report the preparation of a derivative of COS, *N*-benzylidene COS, by alkylation with chlorokojic acid. The chemical structure and physical properties of products were characterized by UV−vis spectroscopy, FTIR, ^{1}H NMR, TGA, SEM, and XRD techniques. Besides, the solubility and the antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, and *Saccharomyces cerevisiae* were also studied. These two chemical groups are expected to supplement each other for their antibacterial activity in the preparation of new effective and environmentally friendly biocides.

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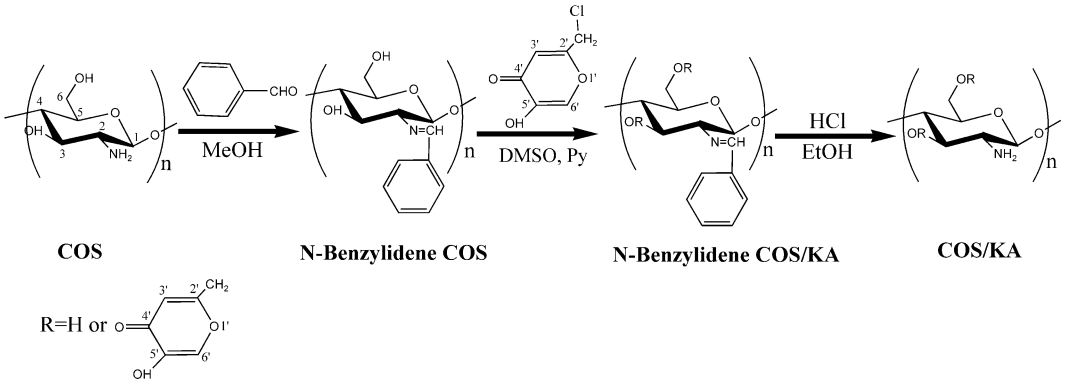

Figure 1. Reaction scheme for the synthesis of COS/KA.

MATERIALS AND METHODS

Materials. Chitosan oligosaccharide (MW = 1 kDa), with a degree of deacetylation of 90%, was made from crab shell and obtained from Zhejiang Jinke Biochemical Co. Ltd. (Zhejiang, China). Kojic acid (2-hydroxymethyl-5-hydroxy-4*H*-pyran-4-one) was purchased from Wuhan Weisheng Biochemical Co. Ltd. (Hubei, China). Chlorokojic acid (2-chloromethyl-5-hydroxy-4*H*-pyran-4-one) was prepared by the reaction of kojic acid with thionyl chloride in chloroform according to the method of Uher and Hudecová.[19] Chlorination of the 2-hydroxymethyl moiety of kojic acid using thionyl chloride at room temperature produced chlorokojic acid, with the ring alcoholic OH being unaffected. Dimethyl sulfoxide (DMSO), deuterium oxide (D_2O), pyridine, and benzaldehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, and *Saccharomyces cerevisiae* used for the antibacterial assay were provided by the School of Food Science and Technology of Jiangnan University. All of the reagents used were of a highly pure grade and were used without further purification, and deionized water was used for all reagent solutions.

Synthesis of Chitosan Oligosaccharides/Kojic Acid (COS/KA). Three chitosan oligosaccharide/kojic acid grafts designated COS/KA1−3 were prepared by the reaction of *N*-benzylidene COS with chlorokojic acid in DMSO and pyridine (Py) (Figure 1). Reaction conditions are summarized in Supplemental Table 1 of the Supporting Information. COS (ca. 6 g) was dissolved in 70 mL of 1% acetic acid and diluted with 120 mL of methanol. Then 6 mL of benzaldehyde was slowly added into the COS solution over 30 min to obtain *N*-benzylidene COS, and the reaction was conducted at 60 °C for 12 h to protect amino groups of COS as Schiff base. After the reaction was completed, the reaction mixture was filtered using a sintered glass filter, and the residue was washed three times sequentially with ethanol and water to remove unreacted benzaldehyde and finally freeze-dried for 24 h. Chlorokojic acid (2.16−1.07 mol) was dissolved in DMSO. By stirring, *N*-benzylidene COS (1 mol) was added to this solution, and then Py was added dropwise at room temperature. Both *N*-benzylidene COS and chlorokojic acid dissolved completely in DMSO, so the reaction was homogeneous in this case. The reaction mixtures were stirred at different temperatures for 6 h and cooled to room temperature. Then an excess of acetone was poured into the DMSO solution, and the product was precipitated. The solid was recovered by filtration, washed repeatedly with acetone, Soxhlet-extracted with ethanol for 24 h, and finally oven-dried at 40 °C for 16 h to obtain *N*-benzylidene COS/KA. Then, the *N*-benzylidene COS/KA was suspended in 1000 mL of 0.25 mol/L hydrochloric acid solution at room temperature for 12 h, followed by filtration and three washings with ethanol and water, respectively, and oven-dried at 60 °C for 12 h to obtain the product.

UV−Vis Spectroscopy. UV−vis absorption spectra of 0.2% (w/v) solutions were obtained using a UV1000 spectrophotometer (Techcomp Ltd., China) in the spectral region of 190−600 nm and with a beam width of 2 nm.

FTIR Spectroscopy. Fourier transform infrared (FTIR) spectrum was recorded on a Nicolet NexuS470 instrument (Nicolet Instrument, Thermo Co., Madison, WI, USA). Samples were prepared as KBr pellet and scanned against a blank KBr pellet background at wavenumber range 4000−400 cm^{-1} with resolution of 4.0 cm^{-1}.

^{1}H NMR Spectroscopy. ^{1}H NMR spectra were obtained on a Bruker AV400 MHz (Bruker, Rneinstetten, Germany). Samples were dissolved in D_2O with tetramethylsilane (TMS) as internal standard.

Thermogravimetric Analysis (TGA). The TGA for the samples was performed on a Mettler Toledo TGA/SDTA851 thermogravimeter (Zurich, Switzerland) at the heating rate of 10 °C/min under N_2 atmosphere in the temperature range of 25−500 °C. STARe software (version 9.01) was used to analyze the thermal stability of the samples.

X-ray Diffraction (XRD). Crystallinity measurements were made using a Bruker D8 Advance diffractometer with an area detector operating at a voltage of 40 kV and a current of 50 mA at Cu Kα radiation of k = 0.154 nm. The scanning rate was 2°/min, and the scanning scope was set from 5° to 80° at room temperature.

Scanning Electron Microscopy (SEM). The morphological characteristics of COS and KA1−3 were checked using SEM (Hitachi S-4800, Hitachi Co., Japan). The samples were attached to SEM stubs using two-sided adhesive tape and spray-coated with gold powder (<10 nm) to improve the conductivity of the surface.

Solubility Test. The solutions of COS, KA, and COS/KA 1−3 were evaluated at a concentration of 5 mg/mL in different solvents including distilled water, anhydrous ethanol, acetone, diethyl ether, ethyl acetate, and glacial acetic acid at room temperature (25 °C).

Antimicrobial Activity. The antibacterial activities of the COS, KA, and COS/KA1−3 were tested using a modified colony counting method on bacteria selected for their resistance to common antimicrobial agents. A representative microbe colony was picked off with a wire loop, placed in nutrient broth, and then incubated at 37 °C overnight. By dilution with sterile normal saline (0.9%) solution, the cultures of *Staphylococcus aureus* and *Escherichia coli* containing 10^7 CFU/mL were prepared and used for the antibacterial test. The bacterial suspension (0.1 mL) was inoculated under aseptic condition into 10 mL of liquid peptone medium containing COS, KA, and COS/KA1−3. Each sample contained test materials at a concentration of 1.0, 1.25, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, or 8.0 mg/mL, whereas a blank without test materials was prepared for comparison. All of the samples were incubated at 37 °C under constant agitation. During incubation, 0.1 mL of each suspension was taken to determine the bacteria count by serial dilution with triplicate plating on agar plates. Then the plates were taken out of the incubator, and the inhibitory activity was calculated.

The inhibitory activity (I) was calculated using eq 1 and expressed as percentage:

$$I\ (\%) = N_1 - N_2/N_1 \times 100 \quad (1)$$

N_1 is the initial cell number (CFU/mL), and N_2 is the cell number after treatment (CFU/mL).

In the determination of the inhibition rate of COS, KA, and COS/KA1−3 on the growth of *Aspergillus niger* and *Saccharomyces cerevisiae* by the same method, the differences were that all of the samples were incubated at 28 °C under constant agitation. The cultures were filtered, the pellet was washed with distilled water and dried at 80 °C to constant weight, and then measured the dry cell weight and the inhibitory activity were calculated.

The inhibitory activity (I) was calculated using eq 2 and expressed as percentage:

$$I(\%) = W_1 - W_2/W_1 \times 100 \quad (2)$$

W_1 is the dry cell weight of *Aspergillus niger* and *Saccharomyces cerevisiae* in the control culture medium, and W_2 is the dry cell weight of *Aspergillus niger* and *Saccharomyces cerevisiae* in the COS, KA, and COS/KA1−3 culture medium, respectively.

The 50% inhibitory concentrations (IC_{50}) against microorganisms were determined for COS, KA, and COS/KA1−3. Fitting of the data was performed by linear regression using the concentration versus percent inhibition. The calculated IC_{50} was submitted to analysis of variance, and the means were compared by the Tukey test ($p \leq 0.05$) using SPSS 20.0.

RESULTS AND DISCUSSION

UV−Vis Spectroscopy Analysis of COS/KA. In the UV−vis spectra of COS, KA, and COS/KA1−3 (Supporting Information, Supplemental Figure 1), broad absorption bands between 200 and 300 nm were observed for all five samples. On the basis of the data reported in the published literature, the broad absorption band of COS might be ascribed to the C═O group.[20] In the UV spectrum of KA, obvious absorption peaks appeared at 219 and 273 nm, ,whereas the UV−vis absorption spectra of COS/KA1−3 obtained at different reaction conditions were quite similar to each other. Two absorption bands of COS/KA1−3 at 207 and 271 nm indicated the presence of a 5-hydroxypyranone group. These bands were assigned to intraligand n→π^* and $\pi\rightarrow\pi^*$ transitions of the chromophoric C═O group,[18,21] indicating that KA had been substituted onto the COS.

FTIR Spectroscopy Analysis of COS/KA. FTIR spectroscopy of COS and COS/KA1−3 is shown in Figure2. From the FTIR spectrum of COS, a characteristic peak at 3422.36 cm^{-1} appeared, and this can be attributed to the −NH and −OH group stretching vibration, as well as inter- and extramolecular hydrogen bonding of COS molecules. The weak band at 2923.83 cm^{-1} was attributed to the −CH− stretch of COS. The characteristic peaks at 1628.76, 1519.37, and 1322.79 cm^{-1} were assigned to the carbonyl stretching $\nu_{C=O}$ (amide I), amine bending δ_{N-H} (amide II), and amide three absorption band of COS, respectively. The absorption band at 1155.97 cm^{-1} was the asymmetric stretching of the C−O−C bridge. In addition, the adsorption band at 1071.52 and 1034.16 cm^{-1} corresponded to the involved skeletal vibration of the C−O stretching and the band at 890.44 cm^{-1} had been assigned to the β (1→4) glycoside linkage. The FTIR spectra of the COS/KA1−3 samples were quite similar to each other. The intense band at 1216.72 cm^{-1} and the shoulder at 911.09 cm^{-1} were broadened and shifted from the corresponding features of chlorokojic acid that could be due to covalent bonding of the kojic acid residues onto the COS.[18] Compared with the NH_2 characteristic absorption band of COS and the absorption band of COS/KA1−3, the characteristic peak at 1519.37 cm^{-1} for the NH_2 group is present in both COS and COS/KA1−3, but the absorption peaks at 1030 and 1074 cm^{-1}, which were attributed to ν_{C-O} of C6−OH and C3−OH of COS, respectively, were substantially weakened. These results indicate that the alkylation reaction mainly occurred not on the amine group but on the alcoholic OH groups of COS.

^{1}HNMR Spectroscopy Analysis of COS/KA. The ^{1}HNMR spectra of COS and COS/KA1 in D_2O solvent are shown in Figure 3. In Figure 3A, the peak at 1.98 ppm existed because of the presence of $-CH_3$ of the N-alkylated glucosamine residue. A singlet at 2.98 ppm was assigned to H_2 of glucosamine and N-acetylated GlcN, and multiplets from 3.24 to 3.94 ppm corresponded to H_3, H_4, H_5, and H_6 of the methine protons of glucosamine and N-acetylated glucosamine.

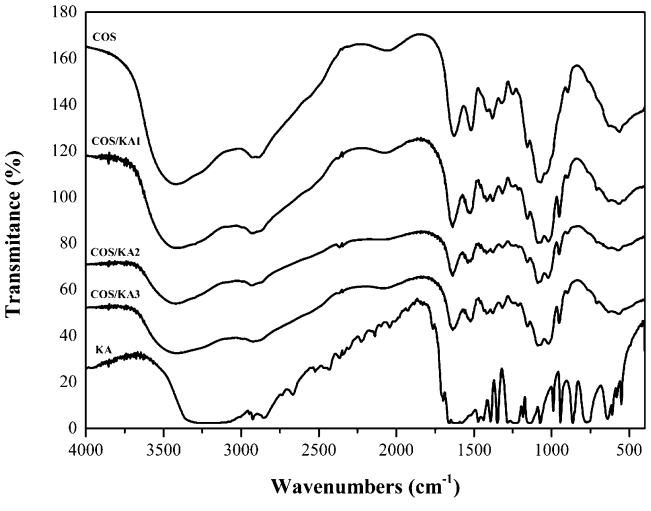

Figure 2. FTIR spectra of COS, KA, and COA/KA1−3.

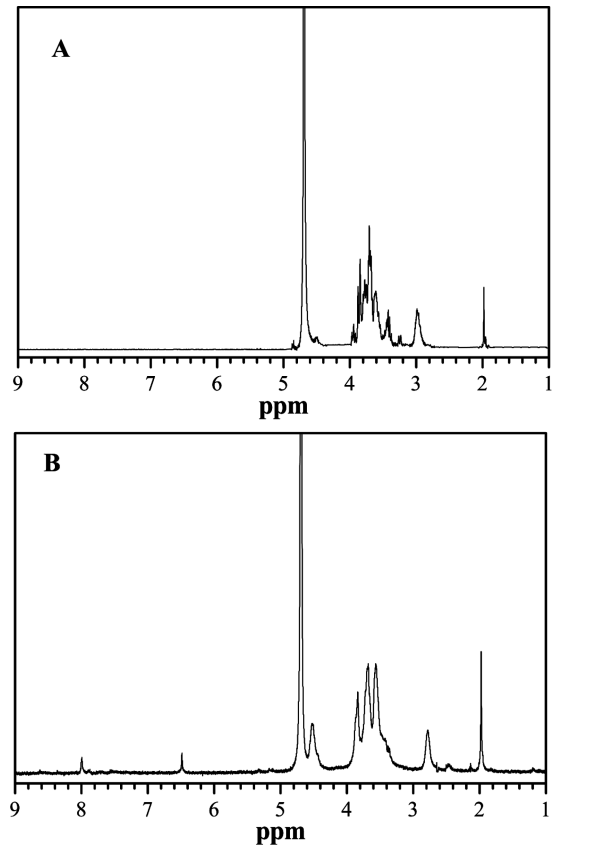

Figure 3. ^{1}H NMR spectrum of COS (A) and COS/KA1 (B).

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The peak at 4.5 ppm was attributed to H_1 of glucosamine, N-acetylated glucosamine. The solvent proton resonates at 4.7 ppm.[22,23] For the ^{1}H NMR spectrum of COS/KA1, the signals of COS protons at 1.98 and 2.98 and from 3.24 to 3.94 ppm did not change significantly their chemical shifts. In Figure 3B, signals of the newly formed three resonance signals appeared at 4.19 (CH_2), 6.59 (H-3′), and 8.07 (H-6′) ppm. These new peaks were assigned to the protons of kojic acid residues bound to the polysaccharide.[10,11] In free kojic acid, the signals due to CH_2, H-3′, and H-6′ have been reported in 4.52, 6.45, and 7.92 ppm regions, respectively.[18] Therefore, compared with kojic acid the signals of aromatic proton H-3′ and H-6′ in COS/KA1 were upfield shifted by 0.14 and 0.15 ppm, respectively, whereas the CH_2 signal was downfield shifted by 0.33 ppm. Hence, the observed shifts confirm that the kojic acid moiety is bound to COS via the CH_2 group.

COS contains two hydroxyl groups in every monosaccharide residue and potentially is able to undergo many reactions of alcohols. One of the most common reactions of alcohols is etherification, which takes place between OH groups of alcohols or between an OH group of an alcohol and a halogen group of an alkyl halide.[24] Aromatic protons of the 4-pyrone ring are much less sensitive to the chlorine substitution than the methylene protons. Hence, the KA moiety is covalently bound to GlcN of COS via the CH_2 group.

TGA of COS/KA. TGA and the first derivative of weight loss (DTG) have been widely used to evaluate the thermal properties of materials and to show the mechanism by which a material loses weight as a result of controlled heating.[25] Figure 4 shows the TGA and DTG curves of COS and COS/KA1−3. Despite some differences in terms of the presence of bound water and residual mass, similar profiles in the curves of mass loss were observed. Both COS and COS/KA1−3 involved two steps of weight loss. In the case of COS, the first stage of weight loss of about 10% between 30 and 100 °C was observed corresponding to the water content. The weight loss of about 47% in the second step was attributed to a complex process including degradation of saccharide rings and disintegration of macromolecule chains in the sample. It had a rapid decomposition between 150 and 320 °C, reaching its peak temperature at 214 °C. As for COS/KA1−3, the first stage, with weight loss of about 6% before 100 °C, was ascribed to the volatile low molecular products and water. The second event, approximately from 160 to 350 °C, refers to a complex process including dehydration of the saccharide rings, depolymerization, and decomposition of the alkylated units of the polymer with weight loss of about 50%.[26] COS/KA1, COS/KA2, and COS/KA3 had similar curves, which showed a maximum degradation temperature (T_{max}) at about 198 °C. This indicates that they have similar thermal stabilities. The T_{max} values of COS and COS/KA1−3 were about 214 and 198 °C, respectively. It was suggested that the thermal stability of COS/KA1−3 decreased compared with COS, which might be attributed to the disruption of crystalline structure. For COS a slight change in crystalline structure could cause significant alteration in thermal stability.[27]

XRD Analysis of COS/KA. The XRD patterns of COS and COS/KA1 are presented in Figure 5. The COS exhibited two peaks at around 2θ = 11° and 2θ = 21°, respectively, characteristic of crystallinity of COS, as has been shown previously.[27,28] In contrast, the peak at 2θ = 11° completely disappeared in COS/KA1, and the peak at 2θ = 21° was wider and weaker, indicating that COS/KA1 was considerably more amorphous than COS.

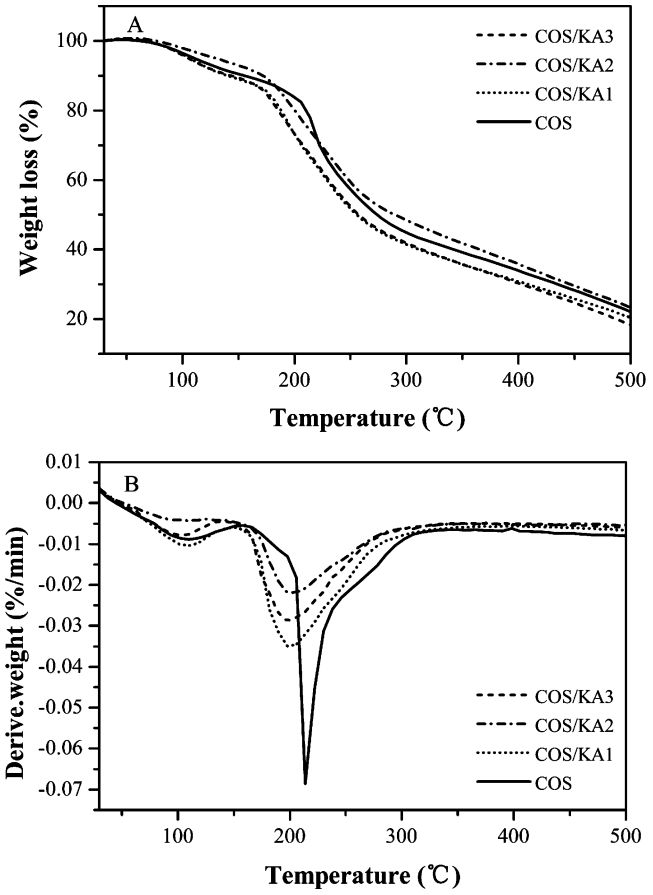

Figure 4. Thermogravimetric curves of COS (solid) and the modified COS: COS/KA1 (short dot), COS/KA2 (short dash dot), COS/KA3 (short dash).

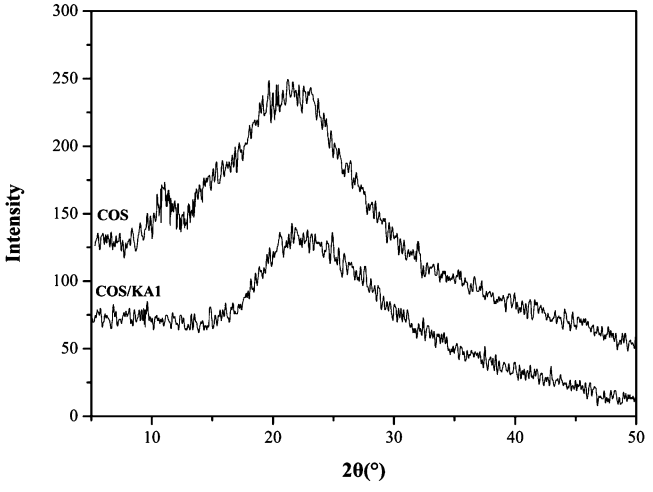

Figure 5. XRD patterns of COS and COS/KA1.

SEM Analysis of COS/KA. Surface morphological studies of COS and COS/KA1 have been investigated using SEM (Supporting Information, Supplemental Figure 2). Because the SEM images of COS/KA1−3 were quite similar, only the image of COS/KA1 is presented here for comparison with COS. The SEM image of COS shows a smooth surface and a more or less spherical shape, because there is stronger

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interaction between the COS molecules, whereas the particles of COS/KA1 were observed to be in the form of irregular flakes and the surfaces of COS/KA1 had many indentations. This could be attributed to effects of drying rate. High drying rates, associated with small particles, usually led to rapid wall solidification, and thus dent smoothing cannot occur.[29] This indicated that the alkylate reaction changed the fiber's microstructure and surface morphology.

Solubility Test of COS/KA. The solubilities of COS, KA, and COS/KA1−3 were investigated in various solvents (Supporting Information, Supplemental Table 1). The results showed that COS did not dissolve in the acetone and diethyl ether, but did dissolve in the other four solvents (distilled water, anhydrous ethanol, ethyl acetate, and glacial acetic acid). KA has better solubility compared with COS. However, COS/KA1−3 could be dissolved in all of the solvents except acetone. In these solvents, COS/KA1−3 showed improved solubility as compared with COS. This can be explained by the contribution of decreased crystallinity confirmed by XRD and TGA.

COS/KA1−3 with different DS values were synthesized by adjusting the molar ratio of chlorokojic acid to COS and the reaction temperature. The DS of derivatives increased when the molar ratio of chlorokojic acid to COS was increased and decreased when the reaction temperature was increased. This might be due to the introduction of a 5-hydroxypyranone group, which disturbed the formation of the ordered structure of COS and hindered the formation of inter- and intramolecular hydrogen bonds. Hence, it was helpful to dissolve.[18]

Antimicrobial Activity Assessment. The antimicrobial effect of chitosans has been reported to be dependent on MW[2,30] and degree of deacetylation,[31] which suggested that chitosans and highly deacetylated COS were more effective at inhibiting the growth of *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, and *Saccharomyces cerevisiae* than COS with low deacetylation. The COS used in this study was highly deacetylated (90%). The antibacterial activities of COS, KA, and COS/KA1−3 against *Staphylococcus aureus*, *Escherichia coli*, *Saccharomyces cerevisiae*, and *Aspergillus niger* are shown in Table 1. At tested conditions, COS/KA1−3 could inhibit the growth of all four tested microorganisms, whereas COS and KA could not inhibit *Aspergillus niger*. The result indicated no noticeable antimicrobial activity when COS and KA were tested against *Aspergillus niger* at concentrations up to 8.0 mg/mL. However, COS/KA1−3 markedly inhibited growth of *Aspergillus niger* with IC_{50} values ranging from 2.15 and 2.46 mg/mL. *Aspergillus niger* belongs to the fungus category, whereas the fungal cell wall contains chitosan. Therefore, *Aspergillus niger* has a certain resistance to the antifungal performance of chitosan.[32] However, COS/KA1−3 showed an excellent antifungal activity and expanded the antimicrobial spectrum.

Table 1. Antimicrobial Activity of COS, KA, and COS/KA1-3 against *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, and *Saccharomyces cerevisiae*

	IC_{50} (mg/mL)				
	COS	KA	COS/KA1	COS/KA2	COS/KA3
Staphylococcus aureus	6.30	6.63	1.04	1.15	1.24
Escherichia coli	7.39	6.04	1.23	1.38	1.58
Saccharomyces cerevisiae	7.37	7.58	1.56	1.63	1.75
Aspergillus niger			2.15	2.37	2.46

It can be observed that the antimicrobial activities of COS/KA1−3 were enhanced with increasing of DS. COS/KA1 (DS = 1.21) displayed the highest antibacterial activity followed by COS/KA2 (DS = 1.13) and COS/KA3 (DS = 0.38) against the four microorganisms with IC_{50} values from 1.04 to 2.15 mg/mL, from 1.15 to 2.37 mg/mL, and from 1.24 to 2.46 mg/mL, respectively. However, COS and KA possessed a weaker antimicrobial activity (IC_{50} from 6.30 to 7.58 mg/mL) compared to COS/KA1−3. The IC_{50} values of COS/KA against antimicrobial activities were 3−7 times lower than those of KA and COS. Hence, the introduction of a 5-hydroxypyranone group to the COS chain greatly enhanced the antimicrobial activity of the COS/KA.

There were also some differences in the inhibition activity against *Staphylococcus aureus* and *Escherichia coli* for COS, KA, and COS/KA1−3. Both COS and COS/KA1−3 showed higher activity against *Staphylococcus aureus* (IC_{50} = 6.30, 1.04−1.24 mg/mL) than against *Escherichia coli* (IC_{50} = 7.39, 1.23−1.58 mg/mL). On the contrary, KA showed higher activity against *Escherichia coli* (IC_{50} = 6.04 mg/mL) than against *Staphylococcus aureus* (IC_{50} = 6.63 mg/mL). This result accorded with the previous studies[33,34] and may be attributed to their different cell walls. The cell wall of Gram-positive bacteria (*Staphylococcus aureus*) is fully composed of peptide polyglycogen. The peptidoglycan layer is composed of networks with plenty of pores, which allow COS and COS/KA molecules to come into the cell without difficulty and allow more rapid absorption of COS and COS/KA into the cell. However, the cell wall of Gram-negative bacteria (*Escherichia coli*) is made up of a thin membrane of peptide polyglycogen and an outer membrane constituted of lipopolysaccharide, lipoprotein, and phospholipids. Because of the complicated bilayer cell structure, the outer membrane is a potential barrier against COS and COS/KA molecules. Therefore, the samples have different effects on the two kinds of bacteria.

As discussed above, the probable antimicrobial mechanisms of COS/KA are suggested as being, on one hand, the positive charge of the amino group at the C-2 in the glucosamine monomer of COS/KA allows interactions with negatively charged microbial cell membranes that lead to the leakage of intracellular constituents. On the other hand, the 5-hydroxypyranone group was introduced along the molecular chain, which can form complexes with various metal ions. The hydroxyl group at the carbon 5 position acting as a weak acid is capable of forming salts with a few metals such as sodium, zinc, copper, calcium, nickel, and cadmium, which is important in cell membranes. The catechol groups readily interact with phospholipids of the cell membrane subsequently disrupting the microbial membrane and may also denature proteins. Hence, we can deduce that COS/KA showed a synergistic effect against four microorganisms. More work is needed to confirm this hypothesis.

In this study, the COS/KA derivatives with different DS values from 0.38 to1.21 were successfully prepared by alkylation reaction of chitosan oligosaccharide and chlorokojic acid in the presence of DMSO and Py. The product was characterized by UV, FTIR, and ^{1}H NMR, and XRD confirmed that the amino groups of COS reacted with chlorokojic acid to form the alkylate chitosan. TGA showed that the thermal stability of COS/KA was lower than that of COS. COS/KA exhibited an excellent solubility in organic solvents and distilled water. The

antimicrobial activity of COS was significantly enhanced by the kojic acid derivative of COS, and it increased with DS for *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, and *Saccharomyces cerevisiae*. Thus, COS/KA may have potential as an antimicrobial agent in food applications. However, more biological studies are needed to understand their mechanisms of action, to improve their activity by other modifications of those molecules, and to determine their safety for food applications.

■ ASSOCIATED CONTENT

Supporting Information

UV−vis absorption spectra of COS, KA, and COS/KA1−3, SEM image of COS and COS/KA1, and reaction conditions for *N*-benzylidene COS with chlorokojic acid, DS, yield and solubility for the derivatives. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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