Journal of Bioscience and Bioengineering
VOL. 120 No. 3, 335–339, 2015

www.elsevier.com/locate/jbiosc

Effect of kojic acid-grafted-chitosan oligosaccharides as a novel antibacterial agent on cell membrane of gram-positive and gram-negative bacteria

Xiaoli Liu, Wenshui Xia,* Qixing Jiang, Yanshun Xu, and Peipei Yu

State Key Laboratory of Food Science and Technology, School of Food Science and Technology, Jiangnan University, Lihu Road 1800, Wuxi, 214122 Jiangsu, People's Republic of China

Received 12 September 2014; accepted 8 January 2015
Available online 11 February 2015

Our work here, for the first time, reported the antibacterial activity of kojic acid-grafted-chitosan oligosaccharides (COS/KA) against three gram-positive and three gram-negative bacteria. Integrity of cell membrane, outer membrane (OM) and inner membrane (IM) permeabilization assay, alkaline phosphatase (ALP) and glucose-6-phosphate dehydrogenase (G6PDH) assay, and SDS-PAGE assay techniques were used to investigate the interactions between COS/KA and bacterial membranes. The antibacterial activity of COS/KA was higher than those of unmodified COS. The electric conductivity of bacteria suspensions increased, followed by increasing of the units of average release for ALP and G6PDH. COS/KA can also rapidly increase the 1-*N*-phenylanphthylamine (NPN) uptake and the release of β-galactosidase via increasing the permeability of OM and IM in *Escherichia coli*. SDS-PAGE indicated the content of cellular soluble proteins decreased significantly in COS/KA-treated bacteria. Hence, COS/KA has potential in food industry and biomedical sciences.

[**Key words:** Chitosan oligosaccharides; Kojic acid; Membrane integrity; Membrane permeability; Antibacterial activity]

Consumption of food contaminated with pathogenic bacteria or their toxins resulting in foodborne illness has been of vital concern to public health (1). It is well-known that the survival of microorganisms in food is an important problem, which can lead to spoilage and deteriorate the quality of food products (2) or cause infection and illness (3,4). Therefore, it is necessary to find a novel way to reduce or eliminate food-related microorganisms during the shelf life of food products. Consequently, there is considerable research interest in the development of antimicrobial agents in order to prevent the growth of foodborne pathogens or to delay the onset of food spoilage (5–7).

Chitosan oligosaccharide (COS) 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, which is of special interest in agriculture, food industry, environmental engineering, and medicine for it has activities such as elicitors of plant defense (8), antibacterial (9), antiinflammatory activity (10,11), and antitumour agent (12). Many derivatives of COS for food industry application can be obtained by chemically altering it under mild reaction conditions (8). Recently, antibacterial activity of COS and its derivatives with specific functional groups such as arginine, oleoyl, and aminoethyl have attracted much attention due to their nontoxic nature and natural abundance (13,14). Hence, the antimicrobial activity of COS was improved by conjugating a functional group onto it indicating that the conjugation strategy was thought to be a good method for development of novel COS derivatives. Meanwhile, kojic acid (KA) is widely used as a food additive for preventing enzymatic browning of shrimps (15), antimicrobial, and antiviral activities (16). For both COS and KA are good natural food preservatives for improving food quality and safety. In our previous study (17), kojic acid-grafted-chitosan oligosaccharides (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) (Fig.1). *N*-Benzylidene COS was prepared by using COS and benzaldehyde in the presence of acetic acid and methanol, and chlorokojic acid was prepared by the reaction of kojic acid with thionyl chloride in chloroform. The antimicrobial activity results of COS/KA showed that it could inhibit the growth of dammaging agents as *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger* and *Saccharomyces cerevisiae*. In addition, it exhibited an excellent solubility in organic solvents and distilled water. These characteristics could make it a novel potential antibacterial agent in food industry.

Recently, it has been shown that COS has a wide inhibition spectrum for not only gram-positive but also gram-negative bacteria (18,19). It has been suggested that a positive charge on the NH_3^+ group of the glucosamine monomer at pH < 6.3 allows interactions with negatively charged microbial cell membranes that lead to the leakage of intracellular constituents (20,21). Previous antimicrobial activity studies showed that chelating ability of kojic acid, which has a catechol-like function, plays a significant role in its antibacterial activities (22). However, up to now, the exact mechanism of the antibacterial action of COS/KA has not yet been reported. Hence, it is important to make clear the interactive means between COS/KA and cell membrane of bacteria. Thus, as part of our ongoing investigation of biological activity of chitosan derivatives, antibacterial activity of COS/KA was investigated against foodborne pathogens as three gram-positive and three gram-negative

* Corresponding author. Tel./fax: +86 510 85919121.
E-mail addresses: xiaws@jiangnan.edu.cn, lxl525j@126.com (W. Xia).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2015.01.010

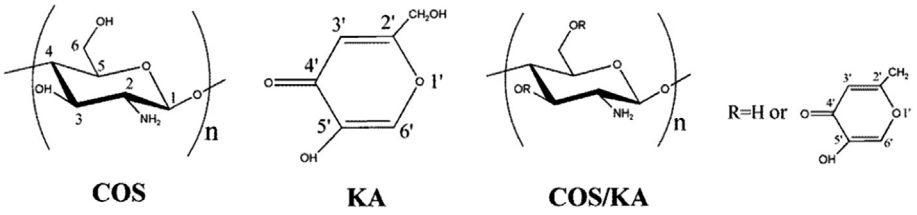

FIG. 1. The structures of COS, KA and COS/KA.

bacteria. In addition, the mode of action of COS/KA on model bacteria (*S. aureus* and *E. coli*) were evaluated using electric conductivity, cell membrane integrity, outer membrane (OM) and inner membrane (IM) permeability assays, the leakage of enzymes from bacteria assays, and SDS-PAGE analysis. This study provides a quantitative understanding of the effect of COS/KA, which could be a useful supports for the further design of much more suitable COS derivatives with structural variants.

MATERIALS AND METHODS

Chemicals Sodium dodecyl sulphate (SDS), acrylamide and *N,N′*-methylenebisacrylamide were obtained from Amresco (Beijing, China). 1-*N*-Phenyl-naphthylamine (NPN) and *O*-nitrophenyl-β-D-galactoside (ONPG) were obtained from Sigma Chemical Co. (St. Loius, MO, USA). 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). All of the reagents used were of a highly pure grade and were used without further purification, and the deionized water was used for all reagent solutions.

^{1}H NMR analysis of COS and COS/KA COS/KA preparation done by following our previous method (17) was confirmed by ^{1}H NMR analysis. To confirm successful synthesis, ^{1}H NMR analysis was conducted. Unmodified COS: ^{1}H NMR (400 MHz, D_2O) δ: 4.5 (1H, H-1), 2.51 (2H, H-Ac), 3.24–3.94 (1H, H-2/6), 4.7 (D_2O). 1.98 ($-CH_3$). COS/KA: ^{1}H NMR (400 MHz, D_2O): a signal of newly formed three resonance signals appeared at δ: 4.19 ($-CH_2$), 6.59 (H-3′) and 8.07 (H-6′). The signals of COS protons at 1.98, 2.98, and from 3.24 to 3.94 ppm did not change significantly their chemical shifts. In this study, the sample with a degree of substitution (DS) 1.21 was chosen for its superior antibacterial activity.

Bacterial strains and media The antibacterial activity of COS/KA against three gram-positive bacterial strains: *Streptococcus pyogenes* (ATCC 12344), *S. aureus* (ATCC 25923) and *Bacillus subtilis* (ATCC 21332), and three gram-negative bacterial strains: *Salmonella typhimurium* (CMCC 50013), *Shigella dysenteriae* (CMCC 51302) and *E. coli* (ATCC 25922) were provided by the Microbiology Laboratory in School of Food Science and Technology, Jiangnan University. Nutrient broth (beef extract 10 g, peptone 10 g, glucose 1 g, NaCl 5 g, H_2O 1000 mL, pH 7.2) and nutrient agar solid medium (beef extract 10 g, peptone 10 g, glucose 1 g, NaCl 5 g, agar 14 g, H_2O 1000 mL, pH 7.2) were used. Nutrient broth was inoculated with a colony formed on nutrient agar medium and shaking cultivated at 37 °C for 12 h. By diluting the cultures with sterile nutrient broth, the cultures of six bacterial containing ~10^7 CFU/mL were prepared and used for further study.

Antibacterial activity Agar well diffusion method was used for the determination of the antibacterial activity. Three circular wells of 8 mm diameter were cut on the agar surface using a sterilized Oxford Cup. Each well was filled with 100 μL of the test agents. The plates were incubated at 37 °C. The diameter of the zone was visually examined and measured using a caliper.

Electric conductivity assay After incubated at 37°C for 4 h, *S. aureus* and *E. coli* cells were harvested by centrifugation at 10,000 rpm for 5 min, respectively. The bacterial cells were washed with sterile 0.9% NaCl. The final cell suspensions were adjusted to 10^7 CFU/mL and mixed with bacteriocidic agents (COS, KA, and COS/KA) to final their concentrations of 5 mg/mL, and then the mixtures were incubated at room temperature and measured for their electric conductivity at different times (23). The experiment without bacteriocidic agents was used as blank control.

Integrity of cell membranes Cells were harvested, washed and resuspended in sterile 0.9% NaCl solution. A certain amount of the bacteriocidic agents was added to each flask, which was separated into several flasks except the control. Samples of 2 mL were removed from the flasks every 1 h, and then immediately filtered with syringe filters (pore size 0.2 μm) to remove the bacteria. Absorbance at 260 nm was monitored with a UV1000 spectrophotometer (Techcomp Ltd., China) (24). Every treatment repeated three times.

Permeabilization of outer membrane OM permeabilization activity of COS and COS/KA was determined by the NPN assay (25). *E. coli* cells grown in LB medium were collected by centrifugation at 10,000 ×*g* for 10 min, washed twice and resuspended in sterile 0.9% NaCl solution. The final cell suspension was adjusted to an absorbance at 420 nm of 1.0 (20,26). To 1 mL volume of bacteria in a quartz cuvette, NPN was added (final concentration: 10 μM). Background fluorescence was recorded using an FLUORMAX-4 fluorescence spectrophotometer, with 1 cm path length cuvettes. Excitation and emission wavelengths were set at 350 and 420 nm, respectively. Then, the above three antimicrobial agents were added. After the addition of 1 mL of a cell suspension, an increase in fluorescence due to partitioning of NPN into the OM was recorded immediately as a function of time until there was no further increase in the emission intensity. Control tests were performed to verify that the enhanced fluorescence was due to NPN uptake by bacteria.

Permeabilization of inner membrane IM of gram-negative bacteria consists of phosphatidyl glycerol and cardiolipin (13). The cytoplasmic β-galactosidase is released as a consequence of change in inner membrane permeability. In this study, IM permeabilization was determined by measuring the release of cytoplasmich β-galactosidase activity from *E. coli* into the culture medium using ONPG as the substrate (27). Logarithmic-phase bacteria grown in nutrient broth were harvested, washed, and resuspended in sterile 0.9% NaCl solution. The final cell suspension was adjusted to obtain an A_{420} of 1.2. COS, KA, and COS/KA solutions (1.6 mL) were mixed with bacteria suspension (1.6 mL) and 30 mM ONPG acetone solution (150 μL), respectively. The production of *o*-nitrophenol over time was determined by monitoring the increase in A_{420} using a 1000 UV–Vis spectrophotometer (Techcomp Ltd., China).

ALP and G6PDH assay Bacterial cells grown as above were harvested, washed and resuspended in sterile 0.9% NaCl solution. A 0.2 mL aliquot of the cell suspension was withdrawn and added to the reaction mixture. Alkaline phosphatase (ALP) and glucose-6-phosphate dehydrogenase (G6PDH) activity were determined using the method described by Malamy and Horecker (28). As for ALP, the reaction mixture contained 0.1 mg *p*-nitrophenylphosphate in 0.5 M Tris–HCl buffer (pH 8), and the reaction was followed at 28°C by measuring the optical density of the suspension at 420 nm. A unit of released ALP activity was defined as the amount of enzyme that produced 1 μM of *p*-nitrophenol-equivalent in 1 min at 28°C. G6PDH activity was determined in a solution containing 0.05 M Tris–HCl (pH 8), 1.0 μM glucose-6-phosphate, 0.4 μM triphosphopyridine nucleotide (TPN), and 0.01 M $CaCl_2$, and the reaction was followed at 28°C by measuring the optical density of the suspension at 340 nm. A unit of released G6PDH activity was defined as the amount of enzyme that reduced 1 μM of TPN-equivalent in 1 min at 28°C.

SDS-PAGE assay Bacterial cells were prepared as described above. Samples containing *S. aureus* and *E. coli* (each approximately 10^7 CFU/L) in nutrient broth were incubated with 5 mg/mL COS, KA, and COS/KA at 37°C, respectively. Controls were run without bacteriocidic agents. Aliquots of 1.5 mL were withdrawn for *S. aureus* and *E. coli*, respectively, and then centrifuged. The pellet was subjected to SDS-PAGE analysis according to Laemmli (29). The SDS-PAGE was performed with a 4% stacking gel and a 10% separating gel followed by Coomassie brilliant blue staining.

Statistical analyses All experiments were carried out in triplicate, and average values with standard deviation were revealed. The data collected in this study were expressed as the mean values ± standard deviation, and significant differences between the two groups were examined using t-test. A P value < 0.05 denoted the presence of a statistically significant difference.

RESULTS AND DISCUSSION

Antibacterial activity The antibacterial activity of unmodified COS, unmodified KA, COS in the coexistence of KA (COS + KA) (molar ratio: 1:1), and COS/KA against three gram-positive bacteria and three gram-negative bacteria are shown in Table 1. The activity appeared to vary among the tested bacteria. The unmodified COS, unmodified KA, and COS + KA showed the inhibition zone