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Chitosan inhibits enterotoxigenic *Clostridium perfringens* type A in growth medium and chicken meat

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Clostridium perfringens is a spore-forming bacterium and a major cause of bacterial food-borne illness. In this study, we evaluated the inhibitory effects of chitosan against spore germination, spore outgrowth and vegetative growth of *C. perfringens* food poisoning (FP) isolates. Chitosan of differing molecular weights inhibited germination of spores of all tested FP isolates in a KCl germinant solution containing 0.1 mg/ml chitosan at pH 4.5. However, higher level (0.25 mg/ml) of chitosan was required to effectively arrest outgrowth of the germinated *C. perfringens* spores in Tripticase-yeast extract-glucose (TGY) medium. Furthermore, chitosan (1.0 mg/ml) was bacteriostatic against vegetative cells of *C. perfringens* in TGY medium. Although chitosan showed strong inhibitory activities against *C. perfringens* in laboratory medium, higher levels (2.0 mg/g) were required to achieve similar inhibition of spores inoculated into chicken meat. In summary, the inhibitory effects of chitosan against *C. perfringens* FP isolates was concentration dependent, and no major difference was observed when using different molecule weight chitosan as an inhibitor. Our results contribute to a better understanding on the potential application of chitosan in cooked meat products to control *C. perfringens*-associated disease.

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1. Introduction

Clostridium perfringens is a Gram-positive, anaerobic, spore-forming bacterium causing histotoxic and gastrointestinal (GI) diseases in humans and animals (McClane, 2007). *C. perfringens* type A producing *C. perfringens* enterotoxin (CPE) is the causative agent of *C. perfringens* type A food poisoning (FP), which currently ranks as the 2nd most commonly reported bacterial food-borne outbreaks in the United States (Hoffmann et al., 2012; Lynch et al., 2006; Scallan et al., 2011). This pathogenic bacterium is a concern to the food industry due to its ability to produce metabolically dormant spores that are highly resistant to various stresses related to food preservation approaches (Li and McClane, 2006a, 2006b; Paredes-Sabja et al., 2007; Sarker et al., 2000). Spore resistance properties facilitate the survival of FP isolates in processed meat and poultry, which are the products most commonly implicated in *C. perfringens* FP outbreaks (McClane, 2007). When growth conditions in food items are favorable, these heat-resistant spores can germinate and outgrow into vegetative cells, reaching high viable cell numbers {~10^6 colony forming Unit (CFU)/g} (McClane et al., 2013). After the food is consumed, viable cells that survived stomach acidity will sporulate in the GI tract, which leads to CPE release and subsequent GI illnesses (McClane, 2007).

Currently, the food industry is interested in developing novel bacterial spore-inactivation strategies to meet the consumers' demand for natural, minimally processed and organic products. Alternative technologies include applications of natural antimicrobial agents in foods in order to maintain food safety, food quality, extended shelf life by controlling microbial spoilage and foodborne pathogen contamination. Chitosan is a natural carbohydrate polymer derived from the deacetylation of chitin, a main component of exoskeletons of crustaceans, insects, and the cell wall of fungi. Different production methods are used to produce chitosan with different chemical properties, which potentially reflect variations in the antimicrobial activity (No and Meyers, 1995). Chitosan shows a broad-spectrum antimicrobial activity against

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both Gram-positive and Gram-negative bacteria, and fungi. Numerous factors may affect chitosan's antibacterial activities; most significantly among others are molecular weight (MW), degree of deacetylation (DD), physical state and pH (Kong et al., 2010). Most of the commercially available chitosan is shrimp-derived and is used as a food additive or preservative, and as an active packaging material (Friedman and Juneja, 2010; Kong et al., 2010; Su et al., 2009). It has been approved as a food additive in Korea's and Japan's generally-recognized-as-safe list (Kong et al., 2010). Intensive research has been focused on the application of different forms of chitosan including films, coatings, and nanochitosans, in different food categories (Friedman and Juneja, 2010; Su et al., 2009). However, information on its effect on bacterial spores, especially *C. perfringens* spores, and its possible application in poultry products is limited. Moreover, the fundamental knowledge of whether chitosan inhibits initiation of spore germination or prevents outgrowth of germinated spores remains unclear.

The objectives of this study were to examine the effects of chitosan against germination, outgrowth and vegetative growth of spores of the enterotoxigenic *C. perfringens* type A. Furthermore, the application of chitosan as an antimicrobial agent to control the germination and outgrowth of *C. perfringens* spores in cooked chicken meat during storage at extremely abusive temperature was also examined.

2. Material and methods

2.1. Bacterial strains and growth conditions

The *C. perfringens* isolates used in this study consisted of three type A FP isolates (SM101, NCTC10239, and E13) (Sarker et al., 2000). All stock cultures were maintained in cooked meat medium (Difco, BD Diagnostic Systems, Sparks, MD, USA) and stored at −20 °C. Bacteria were revived by inoculating the cooked meat stock culture into fluid thioglycollate (FTG) medium (Difco) and incubating overnight at 37 °C. TGY broth (3% trypticase, 2% glucose, 1% yeast extract, and 0.1% L-cysteine) was used for *C. perfringens* vegetative growth and spore outgrowth,as previously described (Paredes-Sabja et al., 2008b).

2.2. Spore preparation and purification

Spores of three *C. perfringens* type A strains (SM101, NCTC10239, and E13) were prepared and purified as described previously (Akhtar et al., 2008; Paredes-Sabja et al., 2008a). Briefly, the stock cultures of *C. perfringens* were inoculated into 10 ml FTG and incubated overnight at 37 °C. Then, 0.4 ml of an overnight FTG culture was inoculated into 10 ml FTG medium and incubated for 8 h at 37 °C. Next, 0.4 ml of 8-h FTG culture was inoculated into 10 ml Duncan-Strong sporulation medium (Duncan and Strong, 1968) and its sporulation capacity was confirmed under phase contrast microscope after incubation at 37 °C for 24 h. Large amounts of spores were prepared by scaling up the aforementioned procedure. Spores were purified by repeated washing and centrifuging with cold sterile distilled water until microscopic examination demonstrated that the suspensions were >99% free of sporulating cells, cell debris, and germinated spores. Purified spores were suspended in sterile distilled water to obtain an optical density at 600 nm (OD_{600}) of ~6, and stored at −80 °C until use.

2.3. Preparation of chitosan solutions

Three stock solutions of chitosan of low MW (50–190 kDa), medium MW (190–310 kDa), and high MW (310–375 kDa), with 75–85% deacetylation, were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Chitosan stock solutions (10 mg/ml) were prepared in 10 ml of 1% (v/v) acetic acid, according to the previously described protocol (Cruz-Romero et al., 2013; Li et al., 2010). These solutions were then filter sterilized (0.45 μm, Millipore, Bedford, MA, USA) and stored at 4 °C. The stock solutions were used within a month of preparation. The stock solutions were then diluted as indicated in the results for each individual experiment.

2.4. C. perfringens spore germination in the presence of chitosan

The purified spore suspensions in water at a final OD_{600} ~ 1.0 were heat activated at 80 °C for 10 min prior to germination as previously described (Paredes-Sabja et al., 2008b, 2009), and then cooled in a water bath at room temperature for 5 min. To examine the ability of chitosan to induce *C. perfringens* spore germination, heat-activated spores of strain SM101 were incubated with sterile distilled water as a control or with various concentrations (0.1, 0.5 or 1 mg/ml) of low molecular weight (LMW), medium molecular weight (MMW), or high molecular weight (HMW) chitosan (suspended in 1% acetic acid and adjusted to pH 6.0). To test the inhibitory effect of chitosan on spore germination, heat-activated spores of strain SM101 were incubated with pre-warmed 100 mM potassium chloride (KCl) in 25 mM Tris-HCl (pH 6.0) alone (control) or supplemented with (0.1 mg/ml) LMW, MMW, or HMW chitosan. To evaluate effect of pH on spore germination inhibition, the final pH of germinant solutions with or without chitosan were adjusted to the desired pH ranging from 3.5 to 6.5. Spore germination was routinely monitored by measuring OD_{600} of the spore-germinant solutions (Smartspec 3000 spectrophotometer; Bio-Rad Laboratories, Hercules, CA), in which OD_{600} is decreased by ~ 60% upon complete spore germination (Paredes-Sabja et al., 2008b). The extents of spore germination were also confirmed by phase-contrast microscopy in which germinated spores lose refractility and become phase dark. Germination rates were calculated as percentage loss of OD_{600} relative to the initial value after 60 min of incubation in KCl with or without chitosan. Results were expressed as percent inhibition of spore germination compared with the control germination (without chitosan) as described previously (Alnoman et al., 2015; Cortezzo et al., 2004). To generalize our data, two additional *C. perfringens* type A FP strains (NCTC10239 and E13) were tested. All germination experiments were performed in triplicates with three different spore preparations for each strain.

2.5. Dipicolinic acid (DPA) release in the presence of chitosan

The release of DPA during *C. perfringens* germination was performed as previously described (Paredes-Sabja et al., 2008b; Udompijitkul et al., 2014). Briefly, spores of FP strain SM101 (OD_{600} of 1.5) were heat activated at 80 °C for 10 min, and then incubated with LMW chitosan (0.1 mg/ml), 100 mM KCl (control) or combination of 100 mM KCl and 0.1 mg/ml LMW chitosan, at 37 °C and at pH 4.5 and 6.0. One milliliter aliquots of germinating solutions were taken at various time periods and centrifuged for 3 min at 13,200 rpm in microcentrifuge tubes, and DPA in the supernatant fluids was determined by measuring absorbance at 270 nm (A_{270}). The DPA content in spore preparations was evaluated by boiling a 1-ml aliquot of germinating spores for 60 min, centrifuging for 5 min in a microcentrifuge, and measuring the A_{270} of the supernatant fluid. Our previous study indicated that ~90% of the material absorbing at 270 nm contained DPA in *C. perfringens* (Paredes-Sabja et al., 2008b).

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