International Journal of Food Microbiology 149 (2011) 218–225

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Inhibition of *Bacillus cereus* spore outgrowth and multiplication by chitosan

Hilde Mellegård [a], Cecilie From [a], Bjørn E. Christensen [b], Per E. Granum [a,*]

[a] Dept. of Food Safety and Infection Biology, Norwegian School of Veterinary Science, P.O. Box 8146 Dep, NO-0033 Oslo, Norway
[b] NOBIPOL, Dept. of Biotechnology, Norwegian University of Science and Technology, NO-7491 Trondheim, Norway

ARTICLE INFO

Article history:
Received 29 April 2011
Received in revised form 14 June 2011
Accepted 18 June 2011
Available online 5 July 2011

Keywords:
Bacillus cereus
Spores
Germination
Chitosan
Molecular weight
Degree of acetylation

ABSTRACT

Bacillus cereus is an endospore-forming bacterium able to cause food-associated illness. Different treatment processes are used in the food industry to reduce the number of spores and thereby the potential of foodborne disease. Chitosan is a polysaccharide with well-documented antibacterial activity towards vegetative cells. The activity against bacterial spores, spore germination and subsequent outgrowth and growth (the latter two events hereafter denoted (out)growth), however, is poorly documented. By using six different chitosans with defined macromolecular properties, we evaluated the effect of chitosan on *Bacillus cereus* spore germination and (out)growth using optical density assays and a dipicolinic acid release assay. (Out)growth was inhibited by chitosan, but germination was not. The action of chitosan was found to be concentration-dependent and also closely related to weight average molecular weight (M_w) and fraction of acetylation (F_A) of the biopolymer. Chitosans of low acetylation ($F_A = 0.01$ or 0.16) inhibited (out)growth more effectively than higher acetylated chitosans ($F_A = 0.48$). For the $F_A = 0.16$ chitosans with medium (56.8 kDa) and higher M_w (98.3 kDa), a better (out)growth inhibition was observed compared to low M_w (10.6 kDa) chitosan. The same trend was not evident with chitosans of 0.48 acetylation, where the difference in activity between the low (19.6 kDa) and high M_w (163.0 kDa) chitosans was only minor. In a spore test concentration corresponding to 10^2–10^3 CFU/ml (spore numbers relevant to food), less chitosan was needed to suppress (out)growth compared to higher spore numbers (equivalent to 10^8 CFU/ml), as expected. No major differences in chitosan susceptibility between three different strains of *B. cereus* were detected. Our results contribute to a better understanding of chitosan activity towards bacterial spore germination and (out)growth.

1. Introduction

Gram positive endospore-forming bacteria in the genus *Bacillus* is a diverse group of species and strains with a ubiquitous distribution in nature, including foodborne pathogens such as *Bacillus cereus* (Kramer and Gilbert, 1989). *B. cereus* can cause two forms of foodborne illness; the diarrhoeal type where enterotoxin is produced during intestinal vegetative growth (Arnesen et al., 2008; Clavel et al., 2004; Granum et al., 1993) and the emetic syndrome, where preformed toxin is ingested (Agata et al., 1995; Ehling-Schulz et al., 2004). Bacterial spores are dormant entities and highly resistant to heat, UV radiation, desiccation, high or low pH values, toxic chemicals and other challenging environmental stresses (Setlow, 2000). These unique characteristics give spore-forming bacteria like *Bacillus* an advantage over non-spore-formers and make them able to survive for instance in food products where other bacteria are killed due to different treatment processes. Inactivation of bacterial spores or inhibition of spore germination and subsequent outgrowth and multiplication reduces the risk of foodborne illness and also food spoilage (Brown, 2000). Spore germination may begin with the addition of a trigger (germinant) and proceeds with a cascade of reactions not requiring direct metabolic energy, like release of pyridine-2.6-dicarboxylic acid (dipicolinic acid [DPA]) and rehydration of the spore core. During outgrowth, which follows germination, initiation of metabolism and macromolecular synthesis occur. This event will normally continue with vegetative growth, if no inhibitors are present (Paidhungat and Setlow, 2002; Setlow, 2003). Outgrowth and further vegetative growth are hereafter denoted (out)growth.

Chitosan is a family of copolymers of glucosamine (GlcN) and its *N*-acetylated analog *N*-acetyl-D-glucosamine (GlcNAc) with varying proportions between GlcN and GlcNAc. Chitosans are usually derived from chitin, the major structural component of exoskeletons of crustaceans (Vårum and Smidsrød, 2005). Following alkaline partial de-*N*-acetylation of chitin to obtain chitosan, chitosan is further depolymerized by chemical or enzymatic methods to produce polymers of different lengths, as reviewed by Aam et al. (2010). Chitosans are shown to exhibit antibacterial activity towards vegetative cells, among them *B. cereus* (Gerasimenko et al., 2004; No et al., 2002; Park et al., 2004; Tsai et al., 2002), and a permeabilizing effect of the biopolymer towards Gram positive and Gram negative cells with the subsequent release of intracellular compounds, such as K^+ ions and nucleotides has been described in several studies (Helander et al., 2001; Liu et al., 2004;

* Corresponding author. Tel.: +47 22 96 48 45; fax: +47 22 96 48 50.
E-mail address: pereinar.granum@nvh.no (P.E. Granum).

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doi:10.1016/j.ijfoodmicro.2011.06.013

Mellegård et al., 2011; Raafat et al., 2008a, 2008b). The prevailing theory on mode of action towards bacteria is therefore membrane perturbation, but there is still little information on the molecular mode of action and bacterial responses to chitosan exposure. There is also a lack of knowledge on chitosans as potential inhibitors of bacterial spore germination and subsequent outgrowth and multiplication. In addition, where chitosan activity on spores is studied, the chitosans applied are often not defined in terms of macromolecular characteristics (molecular weight (MW) and fraction of *N*-acetylation (F_A)), and critical properties of the test solution, such as pH, may not be reported. In a recent study, we found that the antibacterial activity of chitosans towards vegetative cells varied widely with molecular weight and acetylation and also bacterial test species and strains (Mellegård et al., 2011).

F_A of chitosans and pH of test solutions will affect the antibacterial activity as they determine the proportion of charged amino groups (Vårum and Smidsrød, 2005). There is general agreement on increasing antibacterial activity against vegetative cells with decreasing F_A and pH, as the net positive charge of chitosan polymers increases (Chung and Chen, 2008; Devlieghere et al., 2004; Tsai et al., 2002; Tsai and Su, 1999; Wang, 1992). Most commercially produced chitosans have F_A values below 0.2–0.3 (Vårum and Smidsrød, 2005). The pK_a value of chitosan is reported to be between 6.2 and 7.0, depending on the chitosans applied and the test conditions (Anthonsen and Smidsrød, 1995; Domard, 1987; Sorlier et al., 2001; Strand et al., 2001a), and a majority of the amino groups at the C-2 position in the GlcN units will then be protonated at a pH below the pK_a, according to general acid-base theory (Carey, 2006). It is therefore crucial that the antibacterial assays are conducted at a pH below the pK_a of chitosan. The molecular weight (MW) also affects chitosan activity by influencing the degree of interactions between the positively charged chitosans and negatively charged bacterial surfaces (Strand et al., 2001b). There is, however, no consensus in literature on which MW is the most antibacterial. Kumar et al. (2007), for example, found chitosans of low MW to be more active compared to chitosans of high MW, while other authors reach the opposite conclusion (Jeon et al., 2001; No et al., 2002; Li et al., 2010). Also, minimum inhibitory concentrations (MICs) of chitosan towards vegetative cells of different bacteria vary widely. For *B. cereus*, for example, this is reported to range from 0.2 to 12.5 mg/ml (Chen et al., 1998; No et al., 2002; Park et al., 2004; Tsai et al., 2002). Based on the findings in our recent study, we have suggested that much of this variation is due to insufficiently defined chitosans and therefore different macromolecular properties (Mellegård et al., 2011).

As mentioned before, the literature is scarce on the impact of chitosan on bacterial spores, germination and (out)growth. It is also not established whether chitosan inhibits initiation of spore germination or prevents (out)growth of germinated spores, in studies where a reduction in viable cells from spores is detected. To address these issues, we prepared batches of water-soluble chitosans defined in acetylation and molecular weight and tested their activity upon *B. cereus*. Optical density assays were used to evaluate the impact of different chitosans, concentrations, initial spore numbers and test strains on bacterial spore germination, outgrowth and growth. Next, the influence of chitosan on germination was evaluated in a calcium dipicolinic acid (Ca-DPA) assay.

2. Materials and methods

2.1. Experimental strains

There were three different strains of *B. cereus* included in this study. *B. cereus* ATCC 14579 (the type-strain) was obtained from the American Type Culture Collection and is an enterotoxin-producing strain, as is also *B. cereus* strain NVH 0075/95, involved in a large foodborne outbreak in Norway (Lund and Granum, 1996). The emetic toxin-producer, *B. cereus* F4810/72, was originally isolated from human vomit (Taylor and Gilbert, 1975).

2.2. Preparation of chitosan solutions

Chitosans with $F_A = 0.01$ and 0.16 were obtained from FMC NovaMatrix (Norway), and chitosan with $F_A = 0.48$ was obtained from Advanced Biopolymers AS (Norway). The samples were converted into water-soluble hydrochloride salts (chitosan-HCl) as described by Anthonsen et al. (1993) and partially depolymerized with nitric acid (Allan and Peyron, 1989) to obtain different DP ranges (DP = degree of polymerization = number of sugar residues per chain). Reduction of the degraded samples with $NaBH_4$ was performed (reduction of terminal 2.5-dehydro-D-mannose) and DP values determined on basis of size-exclusion chromatography with on-line multi-angle laser light scattering (SEC-MALLS) as previously described in Christensen et al. (2008). Data were processed and number and weight average molecular weights (M_n and M_w, respectively) obtained as reported by Mellegård et al. (2011).

Stock solutions of depolymerized chitosans of 4 mg/ml were prepared in Milli-Q grade at 4 °C overnight and adjusted to pH 4.0–4.5 before filtering (0.45 μm), aliquotation and storage at −20 °C. An overview of the chitosans included in this study is found in Table 1. Note that in the following we will refer to the different chitosans by names A–F from this table.

2.3. MIC and MBC determinations

A stock solution of 4 mg/ml of chitosan C (Table 1) was twofold serially diluted in MQ water in sterile 96-well round-bottom microtiter plates (Nunc, Roskilde, Denmark). *B. cereus* ATCC 14579 was grown to an optical density at 600 nm ($OD_{600\ nm}$) of 0.5 in Iso-Sensitest Broth (Iso-SB) (Oxoid, Hampshire, England) containing 10 mM 4-Morpholineethanesulfonic acid (MES) (Sigma-Aldrich, St. Louis, MO) at a final pH of 6.0, and 10^4 to 10^5 CFU were added to each well on the microtiter plate. The microplate was incubated at 37 °C for 20 ± 1 h and minimum inhibitory concentration (MIC) was read as the lowest concentration of chitosan inhibiting visible growth. 100 μl aliquots were plated from the wells onto blood agar plates and incubated at 37 °C for 18 ± 2 h to determine the minimum bactericidal concentration (MBC). The MBC was defined as the lowest concentration reducing the inoculum by ≥99.0%. The MIC/MBC experiments were carried out three times. Since pH is crucial for the antibacterial activity of chitosan, pH was checked to be the same as the starting pH (6.0) at the end of experiments in selected test solutions (pH indicator strips, Merck, Darmstadt, Germany).

2.4. Spore preparations

Sporulation of *B. cereus* ATCC 14579 was performed in maltose sporulation medium (MSM) according to van der Voort et al. (2010) with some modifications. 0.1–1.0% of a Luria-Bertoni broth (Oxoid) overnight culture (30 °C, 225 rpm rotary shaking) was inoculated in MSM without maltose. *B. cereus* NVH 0075/95 and *B. cereus* F4810/72 were sporulated in chemically defined sporulation medium (de Vries et al., 2004). A 1:10 dilution of a four hours culture of brain heart

Table 1
Characteristics of the chitosan samples included in the study[a].

Chitosan	M_w (kDa)	M_n (kDa)	M_w/M_n	DP_n (calculated)	F_A
A	36.0	18.0	2.0	81	0.01
B	10.6	7.5	1.4	38	0.16
C	56.8	30.3	1.9	152	0.16
D	98.3	59.1	1.7	296	0.16
E	19.6	13.9	1.4	70	0.48
F	163.0	87.4	1.9	437	0.48

[a] Abbreviations: M_w, weight-average molecular weight; M_n, number-average molecular weight; DP_n, number average degree of polymerization; F_A, degree of acetylation.