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Short communication

Validation of hot water and chlorine treatments to inactivate pathogens inoculated on mung bean seeds: Influence of the seed production area

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ABSTRACT

The majority of bean sprout-related outbreaks have been associated with *Escherichia coli* O157:H7 and *Salmonella*, and an effective method to inactivate these organisms on seeds prior to sprouting is important to avoid foodborne pathogen diseases. We have previously tested treatment with a combination of hot water and chlorine, and a high bactericidal effect without decreases in the germination ratio was observed on mung bean seeds produced in China. To evaluate the versatility of this treatment, the present study confirmed whether our disinfection treatment can be applied to mung bean seeds produced in another country (Myanmar). A more than 5.0 log CFU/g reduction of *E. coli* O157:H7 was achieved when the mung bean seeds were treated with hot water at 85 °C for 10–40 s, followed by soaking in a 2000 ppm chlorine solution for 2 h; the hot water treatment at 85 °C for 40 s followed by the chlorine treatment completely eliminated *E. coli* O157:H7 from the mung bean seeds. Additionally, a more than 5.0 log CFU/g reduction was obtained for *Salmonella* after the hot water treatment at 85 °C followed by the chlorine treatment. These treatments did not significantly affect the viability and germination of the mung bean seeds, and a sufficient yield for commercial uses was obtained.

1. Introduction

Seed sprouts are consumed in salads and on sandwiches, and their consumption has increased in recent decades due to their nutritional value. However, many foodborne illness outbreaks related to sprouts have been reported. For example, a *Salmonella* outbreak caused by eating bean sprouts occurred in the UK, and 204 patients were reported by November 2010 (HPA, 2010). In 2011, a severe outbreak of Shiga-toxin-producing *Escherichia coli* O104:H4 occurred in European countries, and fenugreek sprouts were suspected as the source (WHO, 2011). In 1996, the largest outbreak of *E. coli* O157:H7 occurred in Japan, with more than 7000 people being affected, and the consumption of radish sprouts was suspected as a cause of this outbreak (Watanabe et al., 1999). In most of the cases, the source of such pathogens as *Salmonella* and *E. coli* O157:H7 is considered to originate from the sprouted seeds, and the high temperature and humidity conditions used for seed germination are suitable for the rapid growth of pathogens (Castro-Rosas and Escartin, 2000; Feng, Churey, Worobo, 2007; Saroj et al., 2007; Taormina & Beuchat, 1999). Accordingly, the decontamination of seeds prior to germination is important for the safety of sprouted seeds.

A common strategy to reduce the microbiological load of sprout seeds is the use of chlorine treatments, and soaking sprout seeds in 20,000 ppm of calcium hypochlorite for 10–20 min before germination has been recommended (National Advisory Committee on Microbiological Criteria for Foods, 1999). However, such a chlorine treatment is not always successful, with the reduction of pathogen levels being only approximately 2.5 log CFU/g (Montville and Schaffner, 2004), and complete bacterial elimination is difficult to achieve using chemical treatments (Jaquette, Beuchat, & Mahon, 1996; Lang, Ingham, & Ingham, 2000). Accordingly, more effective disinfection techniques are necessary to reduce the risk of disease outbreaks associated with sprouted seeds.

Heat treatments are conventional and effective in reducing the bacterial load of seeds. Weiss and Hammes (2005) reported that reductions of more than 6.0 log CFU/g of *Salmonella* and *E. coli* O157:H7 were obtained by a hot water treatment at 80 °C for 2 min Enomoto, Takizawa, Ishikawa, and Suzuki (2002) indicated that the bactericidal effect of a hot water treatment at 85 °C for 9 s is equal to or higher than a chlorine treatment at 20,000 ppm. Bari,

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Enomoto, Nei, and Kawamoto (2010b) reported that the combination of a hot water treatment and dipping in sodium hypochlorite was able to eliminate pathogens on inoculated mung been seeds without decreasing the germination ratio. In particular, a hot water treatment at 85 °C for 40 s and chlorine treatment at 2000 ppm for 2 h achieved the complete elimination of *E. coli* O157:H7 and *Salmonella* from mung bean seeds in laboratory-scale experiments (Bari, Enomoto, Nei, & Kawamoto, 2010a); in addition, a higher bactericidal effect was confirmed at a commercial scale (Bari et al., 2010b). Regardless, most of the mung bean seeds in Japan are imported from a variety of countries. Because the shape and structure of the seeds vary depending on the production area, it is unclear to date whether such a treatment is applicable to the seeds produced in other countries, and this practical information is important for seed producers. Therefore, this study was designed to validate the hot water treatment and combined sodium hypochlorite treatment for mung bean seeds produced in Myanmar.

2. Materials and methods

2.1. Test seeds

In our previous studies, we used mung bean seeds harvested in China (Bari et al., 2010a, 2010b). In this study, the seeds harvested in another country (Myanmar) were used to confirm the ability of the hot water and chlorine treatments to decontaminate seeds.

2.2. Bacterial strains and inoculated condition

Three strains of *E. coli* O157:H7 (CR-3, MN-28 and MY-29) and two strains of *Salmonella* Enteritidis (SE1 and SE2) were used in this study. All of the pathogen test strains were adapted to grow in tryptic soy broth (TSB) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 50 μg/ml rifampicin (TSBR). Plating on media containing rifampicin greatly minimized the interference of naturally occurring microorganisms and facilitated the detection of the test pathogens on recovery media. Each strain of *E. coli* O157:H7 and *Salmonella* was cultured at 37 °C in 40 ml of TSBR. The cells of each strain were collected by centrifugation (3000× *g* for 10 min at 4 °C) and resuspended in 40 ml of sterile phosphate-buffered saline (PBS; pH 7.2). Equal volumes of the cell suspensions were combined to obtain a bacterial cocktail containing 9.0 log CFU/ml. The seeds (1 kg) were soaked in the pathogen suspension and mixed gently using a sterile glass rod for 5 min. After drying, the inoculated seeds were mixed well and stored at 4 °C until experimental use.

2.3. Hot water treatment

The hot water treatment was performed using a pasteurization machine (Daisey Co. Ltd., Saitama, Japan). A 100 g sample of the inoculated seeds were placed in mesh baskets, and the baskets containing the seeds were placed in hot water at 85 °C for 10, 20, 30 and 40 s. The uninoculated seeds were also treated with hot water to evaluate the changes in germination ratios. After the hot water treatments, the seeds were placed in sterilized cold water for 60 s.

2.4. Combined treatment of hot water and chlorine

The mung bean seeds were treated with hot water according to the method described above and subsequently washed with chlorine solutions at 2000 ppm for 2 h. The chlorine solutions at 2000 ppm were prepared using sodium hypochlorite (Nacalai Tesque, Kyoto, Japan). All of the washing solutions were prepared within 30 min of their use. The washing treatments were performed by soaking 100 g of the seeds in 500 ml of chlorine solution in a stainless steel container (15 cm × 15 cm × 10 cm) for 20 min.

2.5. Microbial analysis

A 10 g sample of the mung beans seeds was placed in a stomacher bag, and 90 ml of peptone-buffered water (pH 7.2) was added. The seeds were then pummeled for 60 s. The properly diluted samples were pour-plated in quadruplicate on tryptic soy agar (TSA) or sorbitol McConkey (SMAC) agar plates supplemented with 50 μg/ml rifampicin (TSAR and SMACR, respectively) to determine the population of *E. coli* O157:H7. The TSAR plates and bismuth sulfite agar supplemented with 50 μg/ml rifampicin (BSAR) plates were used to count the *Salmonella* populations. The inoculated enumeration media were incubated at 37 °C for 24 h–48 h before the presumptive pathogen colonies were counted.

2.6. Detection of surviving pathogens in treated seeds

The survival of the pathogens was further tested when the population of the pathogens was less than the detection limit (<1.0 log CFU/g). The homogenized mixture of seeds (10 g) and TSB (90 ml) in a stomacher bag was kept in an incubator at 37 °C for 24 h, and the homogenates (0.1 ml) were plated by spreading. All of the plates were incubated at 37 °C for 24–48 h, and the surviving pathogens were evaluated.

2.7. Determination of the germination percentage

Approximately 100 seeds were placed between two pieces of water-saturated filter paper (diameter of 90 mm; Whatman International Ltd., Maidstone, UK) in a Petri dish. The seeds were stored in the dark at 25 °C for 6 days, and sterilized water was periodically applied to maintain a high-moisture condition. The germination percentage was calculated based on the number of germinated seeds.

2.8. Statistical analysis

The experiments were replicated three times, and the data were expressed as the mean values and standard deviations. Significant differences in the average values were established by the Tukey–Kramer multiple comparison method at a 5% level of significance using SPSS software (SPSS Inc., Chicago).

Table 1
Effect of hot water treatments on pathogen-inoculated mung bean seeds and the germination ratio.

Treatment conditions	Population recovered (log CFU/g)[a]				Germination ratio
	Recovery media				
	E. coli O15 7: H7		*Salmonella*		
	TSAR	SMACR	TSAR	BSAR	
Control	6.0 ± 0.0^{A}	5.5 ± 0.3^{A}	6.6 ± 0.4^{A}	6.3 ± 0.3^{A}	98.0
85 °C 10 s	2.4 ± 0.8^{B}	1.5 ± 0.3^{B}	3.9 ± 0.6^{B}	3.5 ± 0.7^{B}	98.5
85 °C 20 s	ND (3/3)[b]	ND (3/3)	2.6 ± 0.6^{C}	2.2 ± 0.4^{C}	99.3
85 °C 30 s	ND (3/3)	ND (3/3)	ND (3/3)	ND (3/3)	98.0
85 °C 40 s	ND (3/3)	ND (3/3)	ND (3/3)	ND (3/3)	98.7

[a] The population data are represented by the mean of three independent trials and standard deviations ($n = 3$). Within individual columns, the values followed by different letters are significantly different ($p < 0.05$).

[b] ND indicates a level below the detection limit (<1.0 log CFU/g). The number of positive samples following enrichment is provided.