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Short communication

Synergistic effect of low power ultrasonication on antimicrobial activity of melittin against *Listeria monocytogenes*

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ABSTRACT

Since antimicrobial peptides kill bacteria by pore formation in cell membranes, transient pores formed by low power ultrasonication should result in enhancement of antimicrobial activity. Because of its relatively gentle action, low intensity ultrasound is expected to have no adverse effect on food texture. Experiments were conducted for deactivation of pure culture of *L. monocytogenes* using a model system of naturally occurring antimicrobial peptide (AMP) melittin in the absence as well as in the presence of ultrasonication. In the absence of AMP melittin, ultrasonication has very small effect on cell density upto a power level of 40 W. However, at a higher power level of 60 W, a dramatic decrease in cell density was observed which implied cell lysis. At low AMP concentration, low power ultrasonication did not improve the antimicrobial activity. At high AMP concentrations, however, AMP was found to completely inactivate *L. monocytogenes*. The synergistic effect of AMP with ultrasonication was found to be the maximum at AMP concentration of 0.78 μg/ml of melittin. A dramatic decrease in 2 orders of magnitude in cell density was observed for ultrasonication in the presence of 0.78 μg/ml of melittin compared to either ultrasonication alone or AMP action alone.

1. Introduction

Conventional microbial deactivation processes are expensive because they require very high energy inputs to produce products with impeccable safety. Currently, there are two broad fields of microbial deactivation technologies that are commercially used in the industry: (i) thermal processing technologies (Ansari & Datta, 2003; Simpson, Cortes, & Teixeira, 2006), which mainly use energy generated by conventional heat transfer, inductive microwave heating and radio frequency; and (ii) non-thermal technologies (Ansari & Datta, 2003; Lelieveld & Keener, 2007), in which the deactivation of microorganism is achieved by high pressure (Balasubramaniam, Ting, Stewart, & Robbins, 2004), electromagnetic fields (Yeom, McCann, Streaker, & Zhang, 2002, pp. 1–32), or sound waves (Chemat, Zill-e-Huma & Khan, 2011). Ultrasound is a relatively new method of microbial deactivation. Low frequency and high intensity ultrasound may help deactivate microorganisms by thinning the cell membranes as a result of regular oscillations of the bubbles produced by cavitation. The advantages of ultrasound over heat pasteurization include: the minimization of flavor loss and greater homogeneity (Piyasena, Mohareb, & McKellar, 2003). At low intensities, ultrasound can form transient pores which facilitate transmembrane transport of antibiotics. This may explain the synergism of low frequency and low intensity ultrasound with antibiotics in deactivation of bacteria (Ishibashi et al., 2010; Peterson & Pitt, 2000; Qian, Sagers, & Pitt, 1997; Yu, Chen, & Cao, 2012). Because of its relatively gentle action, low intensity ultrasound is not expected to have an adverse effect on food texture.

Microorganisms can also be deactivated by a certain class of antimicrobial peptides (AMPs) at low concentrations that form pores on the cell membranes causing leakage of cell contents and eventual cell death. Many of these peptides are believed to disrupt or permeabilize the cell membrane, leading to cell lysis and death (Gennaro & Zanetti, 2000; Hancock, 1997). If pore formation by AMP and low frequency ultrasound can be combined to enhance the efficiency of microbial deactivation, it can result in a more economical microbial deactivation process. In order to examine this hypothesis, we investigated the synergistic effect of melittin, a model AMP system, and low intensity ultrasound in deactivation of pure culture of *L. monocytogenes* in the absence of other components. Eventhough these results by themselves are not directly applicable to food systems since melittin is not FDA approved and a

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real food system will contain other ingredients, they are useful in demonstrating the applicability of low power ultrasonics to improve the efficiency of microbial deactivation in such systems using AMP.

2. Material and methods

2.1. Bacteria, growth media and melittin

L. monocytogenes F4244 was incubated at 37 °C for 16 h, which was propagated in BHI broth at 37 °C for 16 h prior to experiments.

Melittin from honey bee (*Apis mellifera*) venom was purchased from Sigma-Aldrich (St Louis, MO) as lyophilized powder.

2.2. Plate count

A 0.1 ml portion of *L. monocytogenes* F4244 suspension, which was propagated in BHI broth at 37 °C for 16 h prior to experiments, was diluted until a concentration that is estimated to be about 10^7 cells per ml is reached which was then spread onto a solid BHI agar plate The plates were incubated at 37 °C for 16 h and the total number of colony forming units per milliliter (CFU/mL) on the plate was determined to obtain the viable bacterial cell numbers.

2.3. Source of ultrasound

An ultrasound bath operating at a frequency of from 20 to 100 kHz and different ultrasonic powers of 20–60 W were used for the experiments with/without antimicrobial peptides. For each experiment, two thirds of the bath was filled with suspension (the medium for ultrasound propagation) at 25 °C. The temperature inside the ultrasonic bath was kept constant by circulating cooling water bath system. *L. monocytogenes* F4244 suspension was placed in a 5 ml sterile glass tube over the transducers in the ultrasonic bath for different specified times, microbial concentrations and power inputs.

2.4. Bacteria treatment

Four parallel experiments were performed 1) Control: the bacteria were placed in 5 ml of sterile phosphate buffer for 30 min without any antimicrobial peptide treatment or exposure to ultrasound; 2) the bacteria were exposed to ultrasound without any antimicrobial treatment for 30 min. 3) the bacteria were exposed to antimicrobial peptide treatment alone at a concentration of 0.78 μg/ml for 30 min without exposure to ultrasound: 4) Combined effect: the bacteria was exposed to ultrasound and melittin at a concentration of 0.78 μg/ml for 30 and 60 min. Following the four different treatments viable bacterial concentration in the solution of the four samples were measured by plate count.

3. Results

The bacterial suspension was exposed to three different levels of ultrasonic power. Exposure of bacterial suspension to 20 W ultrasonic power for 30 min resulted in a slight decrease in CFU compared to control (without any ultrasonic treatment) (Fig. 1). For ultrasonic treatment at a power level of 40 W, CFU was found to decrease by a factor of 10 (Fig. 1). For ultrasonic treatment at a power level of 60 W, however, CFU decreased dramatically by a factor of about 10^4 (Fig. 1). The results demonstrate the magnitude of enhanced killing increases with increase in ultrasound power level.

Experiments under five different conditions were carried out to investigate the synergistic effect of ultrasound and antimicrobial

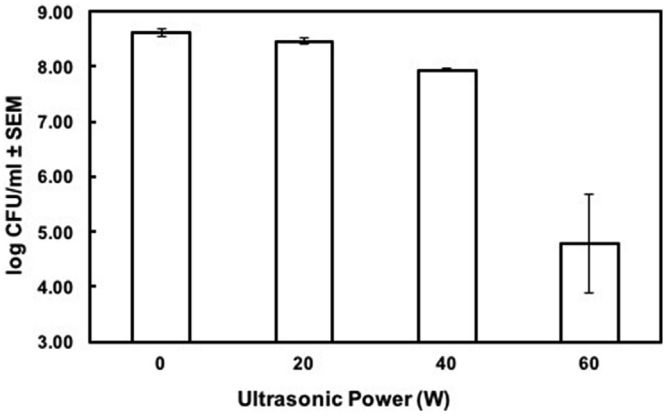

Fig. 1. Bacterial viability (in colony forming units per milliliter) determined by plate count after 30 min at different ultrasonic power levels. Error bars are standard error of the mean (SEM) of three replicates.

peptide on deactivation of bacteria at a power level of 60 W as shown in Fig. 2. When the bacterial suspension was exposed to 60 W ultrasound for 30 min, bacteria were deactivated by a factor of 10^4 compared to control. Treatment of bacterial suspension with antimicrobial peptide melittin at concentrations of 0.39 μg/ml and 0.78 μg/ml also decreased CFU by a factor of 50 and by a factor of 300 respectively. On the other hand, combined ultrasound (60 W) and antimicrobial treatments for 30 min resulted in a decrease in CFU by a factor of 10^4 at 0.39 μg/ml and by a factor of 10^5 at 0.78 μg/ml respectively. In addition, combined ultrasound and antimicrobial treatments of bacterial suspension for 60 min resulted in a complete deactivation of bacteria at 0.78 μg/ml though no further decrease in CFU was observed at 0.39 μg/ml. These results clearly indicate synergism between ultrasound and antimicrobial peptide in deactivation of bacteria which is more pronounced at higher peptide concentration. Results of similar investigation at power levels of 20 W and 40 W are shown in Fig. 3 and Fig. 4 respectively. Results indicate that synergistic effect between ultrasound and antimicrobial peptide in deactivation of listeria is considerably reduced at 40 W whereas no synergism was observed at the lowest power level of 20 W.

Adsorption of melittin onto cell membrane followed by aggregation leads to the formation of surface aggregates which penetrate the cell membrane to form pores. The pore formed by melittin aggregate in cell membrane has hydrophilic inner core as a result of lining of hydrophilic face of the transmembrane peptide on the inside and is toroidal (Leveritt, Pino-Angeles, & Lazaridis, 2015; Mihajlovic & Lazaridis, 2010; Santo, Irudayam, & Berkowitz, 2013; Sengupta, Leontiadou, Mark, & Marrink, 2008; Sun, Forsman, & Woodward, 2015; Zhou, Narsimhan, Wu, & Du, 2014) i.e. some negatively charged phospholipid heads in cell membrane bend to line the interior and partly neutralize the peptide charge. The evaluation of different contributions to free energy of pore has been shown to result in an energy barrier corresponding to a critical pore size (Zhou et al., 2014). The critical pore size has been shown to be smaller at higher melittin concentration (Zhou et al., 2014). Nucleation and growth of pores occur due to thermal fluctuations until they reach critical pore size beyond which the pores grow spontaneously leading to rupture of cell membrane and cell death. Consider an ultrasonication cylindrical probe of radius R and length L . For a frequency f of vibration of the probe with an amplitude x , the pressure wave that is created is $p_0 \sin 2\pi ft$, where the amplitude p_0 is related to power input P by

$$P = \frac{p_0 2\pi RLx}{\tau} = p_0 f 2\pi RLx$$