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Sublethally injured detection on *Staphylococcus aureus* induced damage by pulsed electric field

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ABSTRACT

The effect of pulsed electric fields (PEF) on the reduction of the population and on the occurrence of sublethal injury of *Staphylococcus aureus* was investigated. Flow cytometry (FCM) in combination with PI and cF fluorescent was used for quantitative and real time detection of PEF induced damage on *S. aureus* cells and sublethally injured microbial cells. This work investigated that the occurrence of sublethal injured cells might depend on the electric field intensity. The occurrence of sublethally injured microbial cells in PEF treatment could seriously affect the food safety shelf life of PEF-treated foods. It is effective to synergistically combine PEF with other device to reduce the occurrence of sublethally injured microbial cells and extend the microbiological shelf life of PEF-treated foods.

KEYWORDS

Flow cytometry; Pulsed electric field (PEF); *Staphylococcus aureus*; Sublethally injured cells.

INTRODUCTION

PEF is an effective treatment for inactivating vegetative cells of bacteria and yeast. It has been widely demonstrated that the application of PEF induced the disruption of microbial cell membranes and causes the inactivation of microorganisms[1, 2]. The main applications of PEF for food preservation must be focused on pasteurization. Many studies have been initiated to provide improved knowledge on PEF induced damage on microorganisms by flurescence measurement and electron microscopy[3]. Some recent studies have demonstrated the occurrence of sublethally injured microbial cells under stress of PEF using selective-medium plating technique[4-6]. It may underestimate the numbers of intermediately damaged bacteria, especially for mcirobial cells being able to repair themselves after PEF treatment[7]. Flow cytometry (FCM) in combination with fluorescent techniques offers a powerful tool for analyzing a cell population at the single-cell level, and it can provide quantitative, real-time data acquisition for food and environmental samples[8, 9].

In this study, the PEF resistance and the occurrence of sublethal injury after PEF treatments of *S. aureus* has been investigated. FCM sorting of double stained cells was further successfully used for the analysis of sublethally injured *S. aureus* cells.

METERIALS AND METHODS

Bacterial strain and culture: *Staphylococcus aureus* (JCM 2151) was obtained from China General Microbiological Culture Collection Center. One single colony was maintained on slants of tryptic soya agar (TSA) and inoculated into a flask containing 100ml of sterile tryptic soya broth (TSB) medium (1% tryptone, 0.5% yeast extract, and 0.5% sodium chloride, pH7.0). It was then incubated at 37°C in a well-shaken (180rpm) water bath until OD_{600}=0.5~0.6. Cells were harvested by centrifugation for 5min at 3,000×g, and then washed and adjusted to 10^9CFU/ml in sterile phosphate buffer (10mM, pH7.0) before treatment.

PEF generation and treatments: A bench scale continuous high-intensity PEF system with square-wave pulses was used in this study. The output voltage and pulse width reaches 1~100kV and 1~10μs, respectively. Treatment chamber consisted of two parallel stainless plate electrodes. The distance between the electrodes varied within 1-10cm which set to 1cm in this study. The volume of sample treatment chamber is 1mL, and the pulse frequency is 100Hz here. The temperatures of PEF chambers were adjusted to appropriate temperature by water bath.

Enumeration analysis of viable and injured cells: The bacterial population was determined using plate count agar. PEF treated and control cell suspensions were serially diluted in sterile peptone water. The non-selective agar medium used for enumeration of viable *S. aureus* cells was TSA supplemented with 11% NaCl concentrations that did not affect the growth of untreated cells. The diluted suspensions were serially plated in triplicate LB agar plates. Plates were incubated at 37°C for 48h to detect viable cells and for 72h to detect injured cells before scoring for surviving colonies. The difference between the viable and non-injured cells corresponds to the injured survivors. Each experiment was performed three times on separate days.

Double staining assays: cFDA is a nonfluorescent precursor which could across the cell membranes. Once inside the cell, it undergoes hydrolysis of diacetate groups by unspecific esterases into a membrane-impermeant fluorescent cF[10]. PI can enter into cells with compromised membranes and bind to the DNA and RNA, but it is excluded by viable cells with intact membranes[11]. Untreated and PEF-treated bacterial cells were incubated initially with 50mM cFDA (Life technologies, USA) at 37°C for 10min to allow intracellular enzymatic conversion of cFDA into cF. Cells were then washed with PBS buffer to remove excessive cFDA. Then bacterial cells were incubation with 30mM PI (Becton, Dickinson and Company, USA) for 10min.

FCM analysis: Analysis was performed on a BD Accuri™ C6 flow cytometer (Becton, Dickinson and Company, USA). Cells were delivered at approciately 500 cells/s. cFDA emits green fluorescence at 525nm following excitation with laser light at 488nm (FL1), whereas PI emitted red fluorescense at 620nm (FL2). Green (FL1) and red fluorescence (FL2) of each single cell were measured and analysised by using Accuri™ Cflow software (Becton, Dickinson and Company, USA).

Statistical analysis: All experiments were repeated at least three times. Statistical analysis was applied to all results and a paired t-test was used on results obtained from the same sample and on those normalized results if they were obtained from different samples. All the data was analyzed by Statgraphics5.1 software (Statistical Graphics Corporation, Rockville, MD, USA).

RESULTS AND DISSCUSS

Inactivation and sublethally detection of *S. aureus* cells by PEF treatments

TABLE 1 showed the inactivation of the eletric field intensity on *S. aureus* treated by PEF treatment following by plating onto selective and non-selective media. Clearly, as the electric field intensity increased, the inactivation of *S. aureus* enhanced and all survival data dropped. Non-selective and selective medium supplemented with sodium chloride were

employed, and the survivors at selective medium could be defined as undamaged cells, whereas those at non-selective medium included both undamaged cells and sublethally cells.

TABLE 1: Occurrence of sublethal injury of S. aureus after PEF treatments of different intensities

Electric field intensity	10 kV/cm	20 kV/cm	30 kV/cm	40 kV/cm	50 kV/cm
TSA	0.7±0.3	1.2±0.2	2.2±0.3	3.2±0.2	4.7±0.2
TSA+NaCl	1.4±0.1	2.4±0.2	2.8±0.3	3.4±0.2	4.8±0.3

***TSA: Log10 cycles of inactivation in microorganisms recovered in a TSA non-selective medium.**
*** TSA+NaCl: Log10 cycles of inactivation in microorganisms recovered in a selective medium TSA with 11% NaCl.**
*** PEF treatments under different electric field intensity for 100Hz and 200μs.**

The survived cells in the non-selective and selective media should be the same if no sublethal damage was caused by PEF treatment. As shown in TABLE 1 and figure 1a, a higher level of inactivation was achieved when using the selective medium, indicating that a certain proportion of survivors to PEF treatment were sublethally injured, which could not recover in the selective medium, PEF treatment at 20kV/cm for 200μs resulted in a 2.3 $\log_{10}$ cycles reduction when using the selective medium, higher than the 1.4 $\log_{10}$ cycles reduction obtained using non-selective medium.
Results obtained by plating *S. aureus* in a non-selective media correspond with the procedure used to recover the greatest number of cells. This study investigated sublethal injury casued by PEF treatment. The difference between the Log10 cycles of inactivation calculated in the non-selective and selective media was used to detect the occurrence of sublethal injury after PEF treatments.

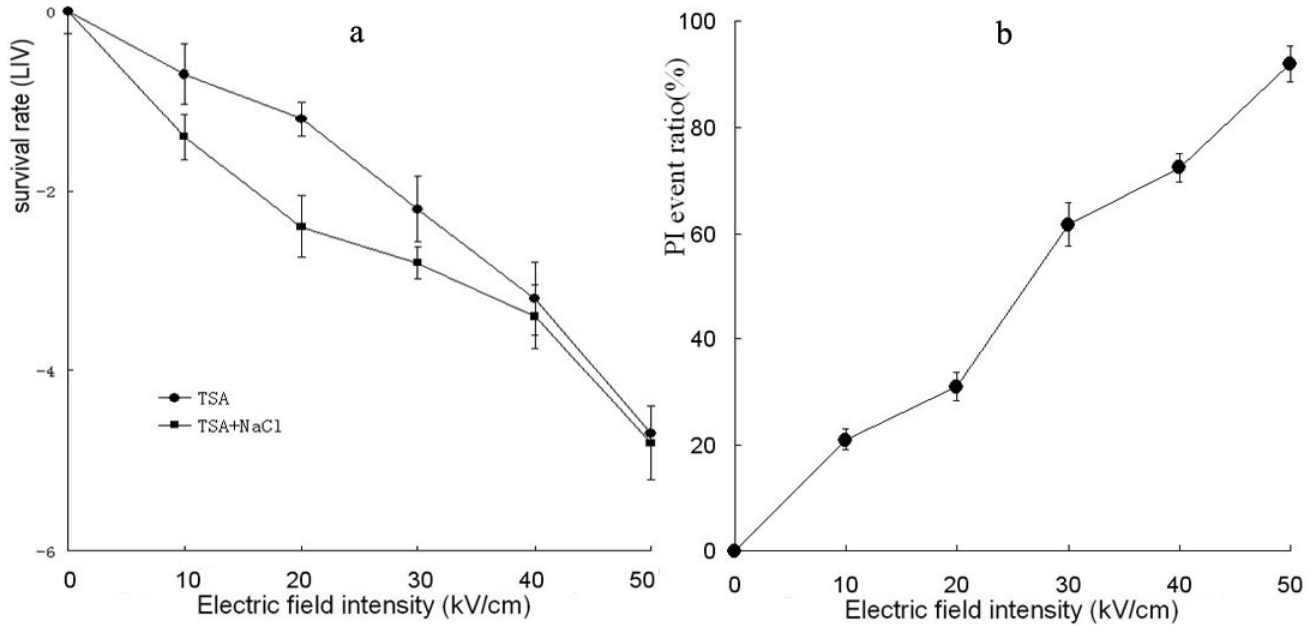

Figure 1: (a) Survival curve of *S. aureus* after PEF treatments at different electric field intensities for 100Hz and 200μs, (b) Percentage of PI-stained cells by FCM after PEF treatments at different electric field intensities for 100Hz and 200μs.

Sublethally injured cells detection by flow cytometry analysis

Flow cytometry offers the ability to physically separate selected cells by cell sorting for further physiological analysis. The dual fluorescence dot plots indicated that four main subpopulations of PEF-treated *S. aureus* cells sorted based on their differential staining characteristics with PI and cF. A population that was not stained by PI or cF appeared in the R1 quadrant (cF^-PI^-). Cells stained only with cF in R2 quadrant (cF^+PI^-), cells stained only with PI in R3 quadrant (cF^-PI^+), and a double stained populations in R4 quadrant (cF^+PI^+).

As shown in Figure 2, most of the cells (90.5%) before PEF treatment were located at R2 quadrant (Figure 2a), demonstrating cells had high esterase enzymatic acitivity and intact membrane. A very small subpopulation (9.4%) appeared in the R1 quadrant representing the debris or lysed cells.

Based on the use of selective and non-selective enumeration media, the cells in R3 and R4 quadrant could be considered as sublethally injured cells, esterase activity not detectable and membrane disrupted cells, respectively. Figure 2 shows percentage of cells in R3 and R4 quadrant after PEF treatments at different electrical field intensities for 200μs. Cells in R3

quadrant steady increased with the increase of electric field intensity from 10 to 50 kV/cm. Compared to subpopulation in R3 quadrant, the sublethally injured cells showed different trends, which greatly increased at low intensities, reached 65% at 30kV/cm, and then decreased at high intensity from 30 to 50 kV/cm. Combined with the results of plate agar counting (figure 1), most of these sublethally injured cells have lost reproductivity or be incapable of repair themselves.

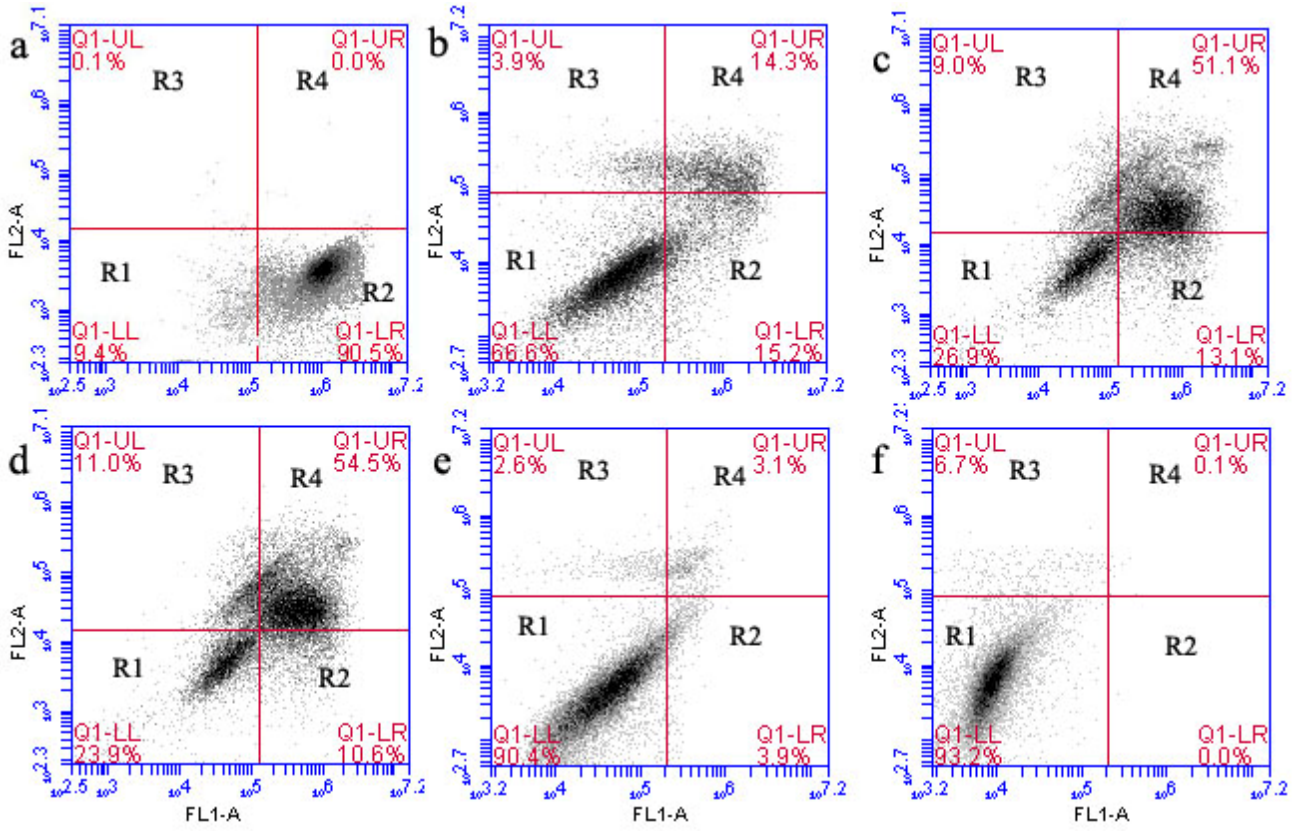

Figure 2: Flow cytometry dot plots of FL1 (green fluorescence collected at 525 nm, CFDA) vs. FL2 (red fluorescence collected at 620 nm, PI) of *S. aureus* cells. The cells were exposed to PEF treatments at 0 (a), 10 (b), 20 (c), 30 (d), 40 (e) and 50 (f) kV/cm for 100Hz and 200μs.

The fluorescence indued by PI is generally associated with cells that have lost their membrane integrity. Flow cytometry histograms of *S. aureus* cells stained with PI before and after PEF treatment to assess effect on reversible and irreversible membrane damage can be found in figure 1b. Figure 1b displayed the percentage of PI-stained *S. aureus* cells exposed to PEF treatments at 0-50kV/cm for 100Hz and 200μs. With the raise of electric field intensity, there was a steady increase in PI fluorescent events, representing the damaged was PI permeable cells induced by PEF. This result could indicate the occurrence of transient pores. The amount of transient pores was dependent on the PEF intensity.

CONCLUSIONS

The mechanism of inactivation of microorganisms by PEF is not clearly understood but almost always includes perturbation of the cell membrane. Data obtained in the study demonstrate that the occurrence of sublethal injury might depend on the electric field intensity. In this work, FCM analysis in combination with fluorescent techniques was employed to detected the sublethally injured *S. aureus* cells and assess PEF-induced damage on membrane and intracellular enzyme activities. The sublethally injured cells were induced by PEF at low electric field (<30kV/cm). The inactivation of intracellular enzyme activity was found involved in mechanisms under stress of PEF. The occurrence of sublethally injured microbial cells in PEF treatment could seriously affect the food safety shelf life of PEF-treated foods. It is effective to synergistically combine PEF with other device to reduce the occurrence of sublethally injured microbial cells and extend the microbiological shelf life of PEF-treated foods.

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