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A Systemic Approach for the Analysis of Microorganisms Inactivated with Supercritical Fluids

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Introduction

Due to the drawbacks of conventional sterilization methods such as thermal processes, alternate microbial inactivation methods applicable to heat-labile materials, fresh foods, pharmaceuticals, and medical equipment are highly demanding. Therefore, the microbial inactivation using SC-CO_2 has been considered as a promising alternative method. Since SC-CO_2 offers high dissolving power and diffusivity, a low viscosity, and easy solvent recovery, it is being considered as an ideal solvent in chemical and biochemical processes. Although SC-CO_2 has recently been studied for inactivating various microorganisms, most of studies have been focused on optimization of inactivation conditions. Therefore, in this study, more systematic approaches such as membrane physiological study, profiling of fatty acids and proteomics by using flow cytometer, GC-MS, and 2D-gel electrophoresis were taken for of *Salmonella typhimurium* (*S. typhimurium*), which is one of the most hazardous foodborne pathogenic microorganisms.

Materials and Methods

After SC-CO_2 treatment conducted at various temperature at a fixed pressure such as 35~55°C at 100 bar and pressure at a fixed temperature such as 80~250 bar at 35°C, each cell suspension (>10^9 cfu/ml) were diluted by phosphate buffered saline (PBS, pH 7.4) to 1×10^8 cfu/ml. One-milliliter of the diluted samples were stained with combinations of three fluorescence dyes: SYTO 9/propidium iodide (PI) or SYTO 9/ethidium bromide (EB). After incubating at 37°C for 15~20 min, the samples were analyzed by flow cytometry. For the analysis of total cellular fatty acids, cells recovered from 5 ml of untreated or treated cell suspension were treated by the modified MIDI protocols: saponification, methylation, extraction, and cleaning. The final extracts were analyzed in scan mode by GC/MS and analyzed by a multivariate analysis, Principal Component Analysis (PCA), using SIMCA-P (Umetrics AB, Sweden). For the proteomic analysis, samples were suspended in a lysis solution and homogenized. The soluble fraction

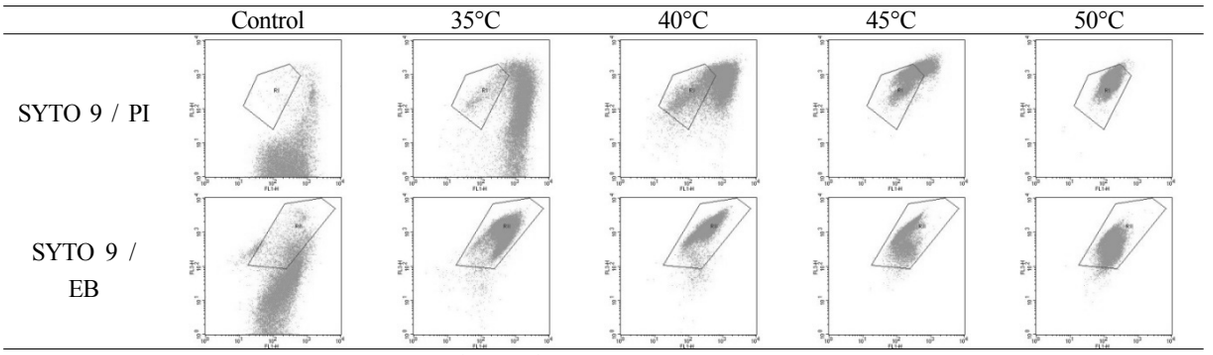

Fig. 1. Flow cytometry dot plot of untreated (control) and treated cells at 35°C, 40°C, 45°C and 50°C. Each treatment was operated at 100 bar for 20 min. The blocked region for SYTO 9 / PI (RI) and SYTO 9 / EB (RII) represent the complete permeabilization and the efflux pump inactivation, respectively.

(crude proteins) was analyzed by 2-dimensional (2-D) gel electrophoresis, and 2-D gels were then silver stained. Quantitative analysis of digitized images of 2-DE was carried out by using PDQuest software. Each spot was normalized by total intensity, and 10 spots with higher ranks were selected and analyzed using MALDI-TOF.

Results and Discussion

Flow cytometric analysis

Flow cytometry offers quick and statistically reliable information about the physiological state of microbial cells. By using fluorescent dyes such as PI and EB, which distinguish the physiological state by specific dyes indicating the membrane integrity and functionality of efflux pump, respectively, the state of *S. typhimurium* treated with SC-CO_2 was analyzed. By the combined staining with SYTO 9 and PI, there was a remarkable decrease in the of membrane integrity as increasing the SC-CO_2 treatment temperature and pressure. The staining with SYTO 9 and EB staining revealed that the efflux pump system was damaged even at a less severe condition compared to the case of the membrane integrity.

Change of cellular fatty acid profiles

As the results of GC-MS analysis of whole cell fatty acids extracted from untreated or treated *S. typhimurium* cells with SC-CO_2, total twenty-nine fatty acids were identified. Among them, major eight fatty acids were over 97% of the total content. The total fatty acid contents of the samples treated with SC-CO_2 were significantly lower than those of the control which was not treated with with SC-CO_2. Moreover, the differences between the treated and the untreated sample were higher as increasing the treatment pressure and temperature. Also, the profiles of the samples treated by high conditions became more complex than the control. For identifying the key fatty acids which were predominantly changed after SC-CO_2 treatment, PCA was chosen as it is an explorative tool that is able to visualize how the fatty acid profiles are changed

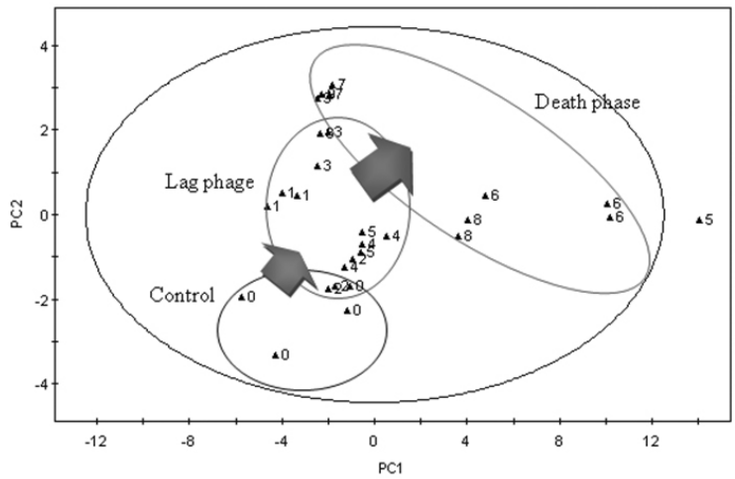

Fig. 2. Principal component analysis plot with component 1 (77%) versus component 2 (10%) based on the cellular fatty acid profiles of *Salmonella typhimurium*. Three clusters were categorized such as the control, 0-2 log reduction, 2-9 log reduction groups. Each number represents SC-CO_2 treatment conditions (pressure and temperature): 0, control; 1, 35°C/80 bar; 2, 35°C/100 bar; 3, 35°C/150 bar; 4, 40°C/80 bar; 5, 40°C/100 bar; 6, 45°C/150 bar; 7, 45°C/80 bar; 8, 45°C/100 bar; 9, 45°C/150 bar. Ellipse: Hotelling T2 (0.95).

in different aspects of the data in the scores and the accompanying loadings. The score plot shows good visual separations among three different survival stages (the control, lag pahse, and death phase). The clusters moved to a certain direction as the inactivation level increased. The good separation among three different groups is not due to the major fatty acids in terms of content, but due to the minor compounds. These results suggest that the SC-CO_2 treatment made their fatty acid profiles to be more complex after the SC-CO_2 treatment. Also, the distance within the cluster of death phase was broader than other clusters. It is probably because various minor fatty acids were more specifically affected by each treatment condition, thus resulting in producing more diverse the fatty acid profiles.

Proteometric analysis of cellular proteins

Any possible changes in the cellular proteins of *S. typhimurium* inactivated by SC-CO_2 were also studied. After the SC-CO_2 treatment at 35°C and 100 bar for 30 min, the total proteins were analyzed

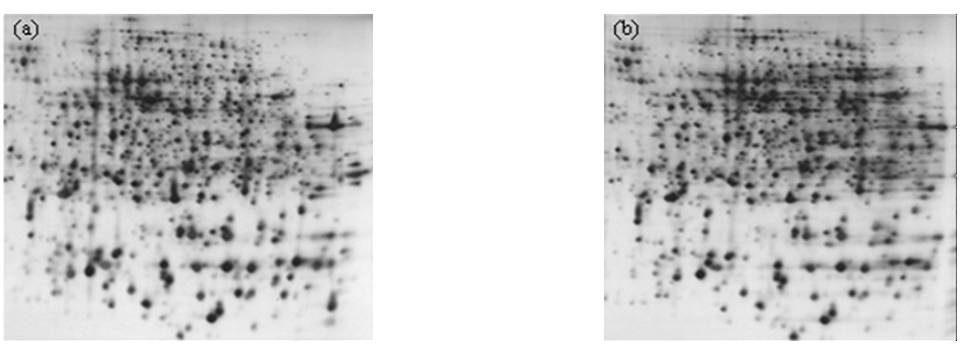

Fig. 3. Two dimentional gel electrophoresis images of cellular proteins of *S. typhimurium* untreated (a) or (b) treated with supercritical CO_2 at 35°C and 100 bar for 30 min.

by 2-D gel electrophoresis. It revealed that significant changes of protein profiles occurred after SC-CO_2 treatment. Overall, the intensity of total changed protein spots were a half of its initial quantity of the proteins of *S. typhimurium*. Also, many of protein spots either disappeared or decreased in intensity, but some protein spots gave increases in intensity.

Conclusions

After the SC-CO_2 treatment, the changes in the cell membrane functions, total fatty acid contents and profiles of proteins occurring in *S. typhimurium* were evident when analyzed by the flow cytometry, GC/MS and 2-DE. Therefore, these results indicate that the systemic analytical methods could be effective and useful tools to get a clear view of chemical and biochemical changes taking place in the SC-CO_2 inactivation of bacteria.