Inactivation of \textit{Bacillus subtilis} Spores in Soybean Milk by Radio-Frequency Flash Heating Treatment

Kunihiko Uemura, Chieko Takahashi, and Isao Kobayashi

National Food Research Institute, Tsukuba, Japan (Uemura@affrc.go.jp)

ABSTRACT

Heat-resistant microorganisms such as \textit{Bacillus subtilis} spores derived from soil may exist in soybean milk and can reduce the shelf life of tofu produced from conventional processing of soybean milk. Conventional high-temperature, long-time heat processing of soybean milk sufficiently inactivates spores of this organism. An apparatus to pasteurize soybean milk using radio-frequency flash heating (RF-FH) was developed. An electrode surface was covered with a 50μm-thick Teflon film, and 27 MHz RF-FH was applied to soybean milk flowing through the electrode unit. No difference was observed between the frequency of 20 kHz and that of 27 MHz regarding the effect of inactivation of \textit{B. subtilis} in saline solution. No difference in inactivation speed of \textit{B. subtilis} by RF-FH at a holding time (1.7 to 5.5 s) was observed between saline solution and soybean milk. However, the inactivation speed of \textit{B. subtilis} in soybean milk by conventional heating was lower than in saline solution. RF-FH could inactivate \textit{B. subtilis} without reference to surrounding soybean milk. Comparative studies revealed that trypsin inhibitor (TI) in soybean milk after RF-FH processing had lower inactivation than TI after conventional heating. A four-logarithm-order reduction of \textit{B. subtilis} spores was realized in the soybean milk by RF-FH at up to 107 °C for 5.5 s.

Keywords: RF heating; Bacillus subtilis spore; trypsin inhibitor

INTRODUCTION

Heat-resistant microorganisms such as \textit{Bacillus subtilis} spores derived from soil may exist in soybean milk and can reduce the shelf life of tofu produced from conventional processing of soybean milk. Conventional high-temperature, long-time heat processing of soybean milk sufficiently inactivates spores of this organism but negatively impacts the gel strength of the resultant tofu, due to protein denaturation.

Ohmic heating is older than microwave heating and was reportedly used to inactivate microorganisms in milk in 1919 [1]. Today, ohmic heating using a frequency of 20 kHz has become a useful technology in the food industry; it has been used to process fish cake since 1990 because of the increased stability and increased energy efficiency. For a long time it was believed that microorganisms in food were inactivated by the electrical effects of ohmic heating. However, Palaniappan et al. [2] reported that ohmic heating did not induce electrical effects for inactivation, and Imai et al. [3] reported on the characteristics of the breakdown of the cell membrane when an electric field was used in the ohmic heating of vegetables, where the voltage in a cell was close to 1 V. Uemura et al. developed high electric field alternating current (HEF-AC) technology that uses an electric field 100 times higher than conventional ohmic heating. The HEF-AC treatment consists of flash ohmic heating in an electrode, holding the temperature in a holding tube, and cooling in a heat exchanger. The HEF-AC technology is a combination of ohmic heating and a high electric field, and involves applying HEF-AC at 20 kHz with an electric field exceeding 2 kV/cm to liquid food between a pair of titanium parallel-plate electrodes. Uemura and Isobe [4] originally designed HEF-AC to inactivate \textit{Escherichia coli} in liquid foods. \textit{E. coli} was dramatically decreased when an AC electric field exceeding 5kV/cm was applied and the temperature of the liquid reached 70°C. It was suggested that the inactivation of \textit{E. coli} was caused by a combination of high-intensity electric field effects and ohmic heating effects. Geveke et al. [5] applied a 20 kHz, 18 kV/cm electric field to \textit{E. coli} in apple juice at the moderately low temperature of 50°C, reducing the \textit{E. coli} to 3 log by the high electric field effect. However, high-voltage pulses were unable to inactivate spores. Uemura et al. [6] used HEF-AC with an electric field of 10 kV/cm on \textit{Bacillus subtilis} spores that were added to orange juice and reduced the number of bacteria by four orders of magnitude by heating the juice at the electrode exit to 120°C. The electric field effect inactivated \textit{B. subtilis} spores less than it did \textit{E. coli}. It was assumed that the inactivation of \textit{B. subtilis} spores by HEF-AC was caused by flash heating produced by HEF-AC. Uemura et al. [7] applied 2.7kV/cm, 20kHz AC to
Alicyclobacillus acidoterestris spores in orange juice and reduced their number by 3 log at an outlet temperature of 125°C and a holding time of 0.9s. This experiment obtained no evidence of the high electric field effect. However, *A. acidoterestris* spores were not inactivated but damaged during the application of flash ohmic heating by HEF-AC, and spores were inactivated 30 times faster than with conventional heating in the holding region between the electrode and the cooling region. Inoue et al. [8] used HEF-AC on various microorganisms, including highly heat-resistant spores that were added to a model liquid, and reduced the bacteria by over three orders of magnitude. With HEF-AC, rapid heating at temperatures over 100°C was required to inactivate B. subtilis spores in a short time.

A comparative study was performed to examine the quality change of orange juice after HEF-AC processing and ultra-high-temperature (UHT) processing that inactivates *B. subtilis* spores in orange juice by four logarithm orders. At least ten percent more L ascorbic acid, hesperidin, limonene, and linalool remained in the orange juice after HEF-AC processing than after UHT processing. However, when HEF-AC technology was applied to liquid food including protein (e.g., soybean milk or cow milk), protein formed scale on the electrode surface due to ohmic heating caused by the HEF-AC [9].

This problem is solved by creating a device in which the soybean milk and electrode do not touch directly, by covering the electrode surface with a thin Teflon film. This is equivalent to an electronic circuit involving a capacitor (the Teflon film and electrodes) in series with a resistor (the liquid food). The resistor is heated by a distributed radio frequency (RF) voltage. The voltage across the capacitor is low when the frequency of the applied alternating current is high. Therefore, radio-frequency flash heating (RF-FH) at frequencies between 3 and 30MHz is used so that the reactance of the capacitor becomes negligible in comparison with the resistance.


This study applied RF heating to soybean milk through a Teflon film to process *Bacillus subtilis* spores in milk and examined the effectiveness of inactivation. Tofu was then made from conventional heat-treated soybean milk, RF-FH heated soybean milk, and another conventional heat-treated soybean milk, and their gel strengths were compared.

**MATERIALS AND METHODS**

**Model food**

Saline solution of 0.2% w/v was autoclaved. The electrical conductivity of the solution (0.832±0.007S/m) at 80°C was equivalent to that of soybean milk (0.755±0.008S/m) at 80°C. It was used as a model food that did not contain protein. The electrical conductivity in an RF range of 3MHz to 30MHz at 80°C was equal to that of the following soybean milk.

**Soybean milk**

The method of making 2L soybean milk was as follows. First, 600g soybean (JA Obihiro Kawanishi, Yukihomare, Japan) was soaked with 3.6L SQ water for 15h, strained using a strainer, and squeezed with 2.1L SQ water by a juicer (Panasonic Co., MJ-M30-Y, Japan). Preheated soybean milk was then produced by heating the raw soybean milk at 105°C for 5min using a high-pressure steam sterilizer (TOMY, LSX-500, Japan).

**Bacillus subtilis spore**

A strain of *Bacillus subtilis* spore (PCI219) was used in this study. Spores were produced on a Nutrient Agar plate (Eiken Chemical Co., Japan) at 37°C for seven days, by which time at least 80% of the cells had sporulated, as determined by microscopic examination. Spores were removed by stirring each tube using a Vortex stirrer after adding 10mL of sterile distilled water. The spore crop was centrifuged at 4000rpm for 10min. The supernatant was decanted, and the pellet was resuspended. The spores were cleaned by repeating this washing step with centrifugation five times. Finally, the pellets were resuspended in a sterile peptone.
solution with 10% glycerin added. The suspension was heated and held at 80°C for 10min and then stored at -80°C under sterile conditions until use.

**RF-FH setup**

The RF-FH setup is illustrated in Fig. 1. The soybean milk in the tank was fed to heat exchanger 1 at a constant flow rate (4.3 mL/s) by a plunger pump (NP-FX-400, Nihon Seimitsu Kagaku, Japan). The temperature of the liquid was increased to 75°C through heat exchanger 1. The liquid then passed through a 7.9mL temperature-holding pipe for 1.9s, a 15.7mL pipe for 3.7s, and a 23.6mL pipe for 5.5s after passing through an electrode of 2.9mL capacity for 0.7s. It then passed to heat exchanger 2 (28mL capacity) for cooling for 6.6s, and finally was exhausted through a pressure adjustment valve to keep the downstream pressure at 0.5MPa. The sensor tip of an optical-fiber thermometer (Reflex-1, Neoptix, Canada) was inserted at the center of the pipe, 2.0cm from the electrode exit, and the temperature at the electrode exit was measured. The processed liquid drained from the exit was sampled after the electrode outlet temperature became stable. The RF power supply fed the electrode with an SWR of less than 1.2, using an impedance-matching device (AT1500DT, Palstar Inc., USA), with the output of the exciter (IC-7200, ICOM, Japan) amplified to a maximum power of 1kW using a linear amplifier (IC-PW1, ICOM, Japan).

**B. subtilis spores counting**

One milliliter of the treated soybean milk was appropriately diluted in Ringer's solution and in standard agar medium, and the number of colonies was determined after pour-plate culturing at 37°C for 48h. Each of the experiments was repeated three times. The CFU counts after each treatment (n) were divided by the initial count (N0) and were logarithmically converted to determine the survival rate. For analysis, the values were averaged, and the standard deviations were calculated.

**Conventional heating**

Soybean milk (10ml) containing 10⁶ cfu/ml *B. subtilis* spores was capped in a test tube. The test tube was heated in the high-pressure steam sterilizer from 80°C to 95, 98 and 100 °C and the temperature was maintained for 0s, 30s, 60s, 120s, 240s. The test tube was dipped in cold water. The test tube was opened on a clean bench, and the content was decimally diluted in Ringer’s solution (DIGO, Wako, Japan). The diluted samples were surface-plated in triplicate onto nutrient agar with an adjusted pH of 4.0. The plates were incubated at 50°C for 48h before presumptive colonies were counted. All experiments were conducted in triplicate.

**Trypsin inhibitor activity assay**

Trypsin inhibitor activity was measured according to the method of Benjakul et al. using BAPNA as the substrate. A solution containing 100μL extracted sample, 200μL (20 μg/mL) trypsin, and 100μL distilled water was preincubated at 37°C for 10min. Five hundred microliters (0.4 mg/mL) BAPNA (prewarmed to 37 °C) was then added to start the reaction. After incubation at 37 °C for 10min, 100μL 30% (v/v) acetic acid was added to terminate the reaction; the sample was then centrifuged. The activity of trypsin was measured...
RESULTS AND DISCUSSION

**Frequency of RF-FH and Teflon film covering the electrode**

The influence of the outlet temperature (80 °C to 115 °C for 1.9 s) on the *B. subtilis* spores in 0.2% saline solution using three kinds of treatment is plotted in Fig. 3. These measurements were conducted using 20 kHz HEF-AC and 27 MHz RF-FH with or without Teflon film (Fig. 2). All the data indicate inactivation increases with increasing temperature at the outlet of the electrode. This coincidence can best be explained as due to inactivation that was caused by internal heating when electricity passed through the liquid.

![Figure 3](image)

Figure 3. Inactivation of *B. subtilis* spores in 0.2% saline solution by selected frequency of AC and with or without insulating Teflon film between the liquid and the electrode.

**Comparison of inactivation speed of *B. subtilis* spores in soybean milk by RF-FH treatment and conventional heating**

An Arrhenius plot was used to compare the inactivation effect at the holding time of RF-FH and that with conventional heating. No difference was observed between the inactivation speed using RF-FH in soybean milk and that in saline solution. The Arrhenius plot using RF-FH in saline solution was in the range of the Arrhenius plot using conventional heating in saline solution. However, the Arrhenius plot for conventional heating in soybean milk was located six times lower than other plots. *B. subtilis* spores in soybean milk were protected from external heating by environmental soybean milk, and the inactivation speed was decreased. However, RF-FH treated *B. subtilis* spores were unaffected by environmental soybean milk. As the activation energies for RF-FH (Ea = 193 kJ/mol) and conventional heating (Ea = 193 kJ/mol) were similar, inactivation using RF-FH may be attributable not to the electric effect but mainly to thermal effects. RF-FH was determined to inactivate *B. subtilis* in soybean milk six times faster than conventional heating at the same holding time. Comparison of inactivation by RF-FH with that by conventional heating indicated that *B. subtilis* spores are killed immediately with a short holding time.
Trypsin inhibitor activity in raw soybean milk

Inactivation of *B. subtilis* spores by RF-FH and by conventional heating is graphed in Fig. 5. These measurements were carried out using RF-FH at 107 °C for 5.5 s and conventional heating at 100 °C for 5 min or 10 min. *B. subtilis* was inactivated one logarithmic order more by RF-FH than by conventional heating at 100 °C for 10 min. RF-FH inactivated trypsin inhibitor activity more than conventional heating (Figure 5). The 100% base level is 13 unit/mg, which includes raw soybean milk. These measurements were conducted using RF-FH and conventional heating. The results indicated that 58% of TI activation remained with RF-FH at 107 °C for 5 s, and 12% of TI activation remained with conventional heating at 100°C for 10 min. It was assumed that this difference was due to the heating time of RF-FH and conventional heating. RF-FH inactivated less trypsin inhibitor than conventional heating.

![Figure 5. Inactivation of *B. subtilis* spores in soybean milk. Figure 6. Inactivation of trypsin inhibitor in soybean milk.](image-url)
CONCLUSION

The present work investigated the inactivation of B. subtilis spores in soybean milk by RF-FH. The results indicated that RF-FH attacked B. subtilis spore in soybean milk with electricity, and inactivated it same as in saline solution. The inactivation speed in soybean milk was up to six times faster than that with conventional external heating. In addition, 50% of trypsin inhibitor activity remained after RF-FH treatment. This result demonstrates that RF-FH treatment inactivated B. subtilis spores without thermal damage to protein, such as trypsin inhibitors.

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REFERENCES