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Pasteurization and changes of casein and free amino acid contents of bovine milk by low-pressure CO_2 microbubbles

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ABSTRACT

The inactivation efficiency of a two-stage system with low-pressure CO_2 microbubbles (two-stage $MBCO_2$) on *Escherichia coli* in physiological saline (PS) and commercial sterilized bovine milk (CBM), and aerobic bacteria in unpasteurized bovine milk (UBM) was investigated. Furthermore, protease-resistance of casein and the free amino acid contents in the treated milk were measured. The number of surviving *E. coli* cells in PS and CBM by the two-stage $MBCO_2$ decreased with increasing pressure in a mixing vessel or increasing temperature in a heating coil. A 3-log reduction in aerobic bacteria in UBM was achieved by two-stage $MBCO_2$ after 1 min with the heating coil at 45 and 50 °C. Casein in UBM treated with only mixing vessel of two-stage $MBCO_2$ was easier to be decomposed by thermolysin, although the casein warmed with the heating coil was difficult to be decomposed by thermolysin and papain. In addition, most of the free amino acid contents in UBM were decreased by the two-stage $MBCO_2$. Therefore, it was suggested that limited inactivation of aerobic bacteria, and change of protease-resistance of casein and free amino acid contents in UBM were induced by two-stage $MBCO_2$.

1. Introduction

A variety of dairy products, including milk, cheese, butter, yogurt are consumed throughout the world. Before it is sold, milk is treated by either low-temperature pasteurization at 63 °C for 30 min or high-temperature, short-time pasteurization at 130 °C for 2 s (Furukawa, 2002). In Japan, dairy products are ordinarily made from milk pasteurized using the low-temperature process, which retains some enzyme activities, because unpasteurized milk cannot be used for the production of dairy products.

Non-thermal pasteurization with pressurized CO_2 has been studied as an alternative to the process employing heat (Damar & Balaban, 2006). Werner and Hotchkiss (2006) reported that pressurized CO_2 at 35 °C, 20.7 MPa and 132 g/kg of CO_2 feeding resulted in 5.36 and 5.02-log reductions of aerobic bacteria and *Pseudomonas fluorescens* added to raw milk, respectively. Erkmen (2000a, b, 2001) reported that inactivation of *Enterococcus faecalis*, *Brochothrix thermosphacta*, and *Escherichia coli* added to whole or skim milk by pressurized CO_2 was attempted, and the inactivation efficiency in whole milk was less than that in skim milk. Hongmei, Zhong, Liao, and Hu (2014) reported that the number of surviving aerobic bacteria, yeast and molds, and coliform bacteria decreased with increasing temperature, pressure, and exposure time. In addition, there are some reports about precipitation of casein in milk by pressurized CO_2 and the characteristic of the casein, and fractionation of α-lactalbumin and β-lactoglobulin from whey protein concentration and whey protein isolate solution by pressurized CO_2 (Hofland, Berkoff, Witkamp, & Van der Wielen, 2003; Hofland, Van Es, Van der Wielen, & Witkamp, 1999; Strange et al., 1998; Tomasula et al., 1995; Bonnaille & Tomasula, 2012; Yver, Bonnaillie, Yee, McAloon, & Tomasula, 2012). Until now, both pasteurization and protein-denaturation by pressurized CO_2 have ever examined in parts.

We recently developed an equipment that pasteurized liquid samples using heat and pressure after feeding microbubbled (MB) CO_2 into the liquid at a temperature lower than 10 °C and a pressure lower than the critical pressure (two-stage $MBCO_2$), and also reported the inactivation of some microorganisms and enzymes using the two-stage $MBCO_2$; the quality of sake and beer treated with the two-stage $MBCO_2$, and the inactivation mechanisms of $MBCO_2$ on *Saccharomyces pastorianus* different from heat sterilization (Kobayashi, Ikeura, Odake & Hayata, 2014; Kobayashi, Ikeura, Odake, & Sakurai, 2014; Kobayashi et al., 2014; Kobayashi & Odake, 2015). In addition, it was shown to decrease the free

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amino acid contents in unpasteurized sake and unfiltered beer by the two-stage $MBCO_2$ in our previous study (Kobayashi, et al., 2014; Kobayashi & Odake, 2015). However, solution containing high protein or high fat like milk have never been treated by the two-stage $MBCO_2$. Therefore, to expand the use of the two-stage $MBCO_2$ as a pasteurization technique for various foods, it is important to apply this method on milk.

In this study, the inactivation efficiency of two-stage $MBCO_2$ on *E. coli* added to physiological saline (PS) and commercial bovine milk (CBM) already pasteurized using a high-temperature short-time process was compared. *E. coli* was also used as test microorganisms, because it was hygiene indicator bacterium and was often used in inactivation experiment of pressurized CO_2 (Damar & Balaban, 2006). Furthermore, aerobic bacteria in unpasteurized bovine milk (UBM) were also inactivated using the two-stage $MBCO_2$, and changes in protease-resistance of casein and free amino acid contents in the treated milk were measured.

2. Materials and methods

2.1. Preparation of E. coli suspension

E. coli K12 (NBRC3301) obtained from Biological Resource Center, National Institute of Technology and Evaluation (Kisarazu, Japan) was inoculated into a test tube containing 10 mL of nutrient broth (Difco, Becton Dickinson, Franklin Lakes, NJ) and incubated at 30 °C of optimum temperature for 16 h. The cells were then collected and washed by centrifugation at low temperature for reducing negative effect on *E. coli* cells (5 °C, 8000 × g, 10 min). The *E. coli* cells were added to PS and CBM (over 35 g/kg milkfat, 34 g/kg protein; Meiji Co. Ltd., Tokyo, Japan) purchased from a local supermarket (1.0×10^6 CFU/mL).

2.2. UBM

UBM (44 g/kg milkfat content, 34 g/kg protein content, 1.2×10^6 CFU/mL of initial number of surviving cells) was obtained from Fuji Animal Farm, Nippon Veterinary and Life Science University (Yamanashi, Japan). The milkfat and protein contents in UBM were measured by the modification of IDF method (IDF (International Dairy Federation), 1981) and report by Finete, Gouvea, Marques, and Netto (2013), respectively.

2.3. Procedure for the two-stage $MBCO_2$

The equipment used for the two-stage $MBCO_2$ treatment is the same as for a previous report (Kobayashi et al., 2014). Ten liters of the sample solution was injected into the mixing vessel (300 mm dia. × 220 mm height, volume 15 L) at 10 °C, the top of the vessel was then tightly closed, and gaseous CO_2 was fed into the headspace of the mixing vessel to set pressures. Next, $MBCO_2$ was generated by sending the sample solution using a circulating pump at 15 L/min and gaseous CO_2 at 2 L/min to an ejector type MB generator. After $MBCO_2$ was generated until the dissolved CO_2 in the sample solution reached its saturation concentration (approximately 5 min), CO_2-feeding and the circulating pump was stopped. The CO_2-saturated sample solution was continuously pumped by a metering pump to a heating coil (5 mm dia. × 5100 mm length, volume 100 mL). The exposure time in the heating coil was set by adjusting the flow rate of the metering pump. The two-stage $MBCO_2$ treatment was performed under the following conditions. The temperature and pressure in the mixing vessel were set at 10 °C and 1 or 2 MPa, respectively, and the temperature, pressure, and exposure time in the heating coil were set at 35, 40, 45 or 50 °C, 4 MPa, and 1, 3, 5, or 10 min, respectively.

2.4. Measurement of surviving E. coli and aerobic bacterial cells

The number of surviving *E. coli* cells in PS and CBM was measured by plating 1.0 mL each of a sample or diluted sample on duplicate nutrient (for *E. coli*, Difco, Becton Dickinson, Franklin Lakes, NJ) and standard plate count (for aerobic bacteria, Nissui Pharmaceutical Co., Ltd., Kyoto, Japan) agar plates, respectively. These plates were incubated at 30 °C (for *E. coli*) and at 35 °C (for aerobic bacteria) for 48 h, respectively. After the incubation, plates with 30–300 CFU were chosen, and the colonies were counted. If there were low numbers of viable cells, colonies on plates from the undiluted sample were counted (Kobayashi et al., 2014). The detection limit was 1 CFU/mL. All experiments were performed in triplicate. Data was presented as the means with standard errors of the results from triplicate experiments.

2.5. Measurement of protease-resistance of casein

The pH of UBM was adjusted to 4.6 with HCl at 20 °C and casein was collected from the UBM by centrifugation (5 °C, 15,000 × g, 15 min). The obtained casein was twice-washed by centrifugation (5 °C, 15,000 × g, 15 min) with deionized water and was freeze-dried.

Protease-resistance of collected casein was measured based on report by Ishikawa, Shimoda, Kawano, and Osajima (1995). The casein was dissolved in 1.5 mL of McIlvaine buffer (pH 7.4) at 10 and 5 g/L for test with papain (EC3.4.22.2, Wako Pure Chemical Industries, Co., Ltd., Osaka, Japan) and thermolysin (EC3.4.24.4, Wako Pure Chemical Industries, Co., Ltd.), respectively. Five hundred microliters of 0.5 g/L papain solution (in McIlvaine buffer solution, pH 7.4) or 0.5 g/L thermolysin solution (in McIlvaine buffer solution, pH 7.4) were added into the casein solution, respectively, and reacted at 37 °C for 10 min with shaking. For stopping the enzyme reaction, 3 mL of 0.4 mol/L trichloroacetic acid solution was added and incubated at 37 °C for 30 min with shaking, and the reacted solution was filtrated. One mililiters of the filtration was mixed with 5 mL of 0.55 mol/L sodium carbonate and 1 mL of fivefold-diluted phenol solution (Wako Pure Chemical Industries, Co., Ltd.) and incubated at 37 °C for 30 min with shaking. The absorbance at 660 nm was measured using a spectrophotometer (U-5100, Hitachi High-technologies Co., Tokyo, Japan). The relative protease-resistance of casein was estimated using the following formula.

$$\text{The relative protease-resistance of casein} = (A_{660} \text{ after treatment}/A_{660} \text{ before treatment}) \quad (1)$$

2.6. Measurement of free amino acid contents

UBM and 30 g/L sulphosalicylic acid solution were mixed at a ratio of 1:1 by volume and refrigerated overnight. The mixture was centrifuged (5 °C, 10,000 × g, 10 min) and then filtered through a 0.22 μm filter. Free amino acid contents in the filtrate were measured using an automated amino-acid analyzer (JLC500-500/V2, JEOL Ltd., Akishima, Japan). All experiments were performed in triplicate. Data was presented as the means with standard deviation of the results from triplicate experiments.

2.7. Statistical analysis

Significant differences were evaluated using the Tukey–Kramer method ($p < 0.01$) or t-test ($p < 0.01$).