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Enhancement of Non-thermal Treatment on Inactivation of Glucoamylase and Acid Protease Using CO_2 Microbubbles

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Abstract

Thermal treatment is usually used for food pasteurization and enzyme inactivation. However, it has an adverse effect on the quality of thermal-sensitive food such as fruit juice, Japanese sake, milk, yogurt and jam. In this study, we presented an alternative method for a non-thermal treatment with at 45 and 50°C for glucoamylase and protease inactivation using pressurized carbon dioxide (CO_2). Twelve liters of enzyme solution (0.004% glucoamylase or 0.015% protease) was fed into a low pressure (2 MPa) CO_2 mixing vessel. CO_2 microbubbles (MB-CO_2) were generated by introducing the mixture through a swiveling microbubble generator. The mixture containing MB-CO_2 was flowed to incubate in a heating coil at various conditions (temperature at 45 or 50°C and pressure 2, 4, or 6 MPa). After incubation, the mixture was sampled at 10, 20 and 30 min from the sampling valve. The relative residual activities of glucoamylase and acid protease were measured by a spectrophotometer at the absorbance of 400 nm (Abs_{400}) and 660 nm (Abs_{660}), respectively. Relative residual activity of glucoamylase with MB-CO_2 treatment at 50°C and 4 MPa was 15.01% whereas 74.83% of glucoamylase activity was found from treatment without MB-CO_2 at same temperature. For acid protease, relative residual enzyme activity with MB-CO_2 treatment at 45°C and 4 MPa was 2.29% whereas that without MB-CO_2 treatment at 45°C was 81.25%. These results suggested that glucoamylase and acid protease could be inactivated effectively at 45 and 50°C present of MB-CO_2.

Keywords : Enzyme inactivation; Carbon dioxide; Microbubbles; Glucoamylase; Protease

Introduction

Enzyme inactivation in various foods and beverages are desired in the food industry. Its activity produced undesirable chemical changes in food attributes; e.g. color, texture, and flavor during storage and distribution [1]. For example, glucoamylase degrades the quality of sake by producing an excessive amount of glucose [2] which causes a bitter taste. Acid protease degrades casein components in milk [3]. Polyphenol oxidase causes enzymatic browning in fruit juice [4] and also degrades the sensory quality of wine during the aging by polymerization polyphenol compounds [5]. Pectin esterase (PE) causes undesired cloudy instability in orange juice [4]. So far, thermal treatment is used widely for enzyme inactivation in the food industry [6,7]. However, thermal treatment (≥65°C) can alter the nutrition and qualities of thermal-sensitive food [7,8] such as fruit juice, Japanease sake, milk, yogurt and jam. Poor solubility of calcium was observed in the thermal treatment of milk [9]. Tanimoto et al. [2] reported that unpasteurized sake treated thermally at 65°C lost its fresh flavor. The negative effect of thermal treatment on the aroma and flavor of beer was also reported [10]. The sensory quality and nutritional content of coconut juice processed by heat treatments also changed [11].

To eliminate the disadvantages of thermal treatment, developments of innovative non-thermal technologies for enzyme inactivation have been encouraged [8]. In the last decade, high pressure carbon dioxide (HP-CO_2) has emerged as a non-thermal treatment which is able to inactivate enzyme activity [12]. It has been applied in both solid and liquid food matrices [11]. The use of CO_2 treatment has become an attractive technique in food processing for enzyme inactivation because it is nontoxic, non-flammable, inexpensive and no residue [11,12]. Furthermore, CO_2 is guaranteed as a substance that can be used safely on food products [11]. Peroxidase (PO) and polyperoxidase (PPO) in red beet and carrot juice were inactivated using HP-CO_2 treatment at 10-30 MPa [13,14]. Yoshimura et al. [15] reported the inactivation kinetics of acid protease and α-amylase with supercritical CO_2 technique (SC-CO_2) at 30 MPa. The inactivation of α-glucosidase, glucoamylase, α-amylase and carboxypeptidase in fresh sake using HP-CO_2 treatment at 2 MPa was investigated [16]. Gui et al. [17] showed that PPO in cloudy apple juice was inactivated by SC-CO_2 at 30 MPa. The inactivation of acid protease and glucoamylase using SC-CO_2 bubbles at 30 MPa were reported [18-20]. However, SC-CO_2 require a high pressure conditions about 10-30 MPa. Furthermore, both HP-CO_2 and SC-CO_2 induced loss of flavor due to the fact that they extract some constituents including phospholipids and hydrophobic compounds [21,22]. Gasperi et al. [23] reported that the HP-CO_2 treatment induced a reduction in the concentration of many volatile compounds (ester and aldehydes) responsible for the observed change in odor and flavor of treated apple juice. In the last decade, microbubbles (MB-CO_2), very fine bubbles with a diameter of 10-50 μm, had also been investigated for its potential as an alternative process for enzyme inactivation [24]. MB-CO_2 was studied at the present for different characteristics from ordinary bubbles because of their reduced diameter size [24]. Some advantages of microbubbles are their highly specific area (surface area per volume) and high stagnation in the liquid phase, which increases the gas dissolution [24,25]. The key differences between ordinary bubbles, micro bubbles and nano bubbles were explained by Takahashi [26]. The ordinary bubbles go up rapidly and burst at the surface whereas nano bubbles (diameter<200 μm), remain for months and do not burst out at once [26]. In the case of microbubbles, they tend to gradually

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decrease in size and subsequently collapse due to the long stagnation and dissolution of interior gases into surrounding water. Furthermore, hydroxyl radicals occur when microbubbles collapse due to the high density of ions in gas-liquid interface just before the collapse [27]. The type of gas used for the generation of microbubbles can also affect the quantity of free radicals occurring [24].

Enzyme inactivation using low pressurized MB-CO_2, which required lower pressure conditions SC-CO_2, have been studied by several researchers [5,28-31]. Kobayashi et al. [28] showed that esterase, esterase lipase, leucine arylamidase, valine arylamidase and acid phosphatase from *Saccharomyces pastorianus* were completely inactivated by MB-CO_2 at 2 MPa and 50˚C. The quality of Japanese sake, which α-amylase, glucoamylase and acid carboxypeptides enzyme were inactivated by MB-CO_2, retained good taste and flavor [29]. Kobayashi et al. [30] reported that score of sweetness and total aroma of MB-CO_2 treated Japanese sake were higher than unpasteurized Japanese sake. Kobayashi et al. [5] reported the inactivation kinetics of PPO using MB-CO_2 and a decrease in decimal reduction time (D value) and activation energy in MB-CO_2 treatment. In this study, we analyzed the capability of glucoamylase and acid protease inactivation at 45-50˚C by using a MB-CO_2 continuous system.

Materials and Methods

Enzyme solution

Glucoamylase and acid protease extracted from *Aspergillus niger* was purchased from HBI enzyme INC., Shiso, Japan. Glucoamylase and acid protease enzyme powder was dissolved in a concentration of 0.004% and 0.015% w/v, respectively. The concentration of sodium acetate buffer (pH 4) was 10 mM.

Enzyme inactivation with MB-CO_2 treatment

The MB-CO_2 continuous system used in this study is shown in Figure 1. This system has been previously reported [5,30,31]. Twelve liters of glucoamylase (0.004% w/v) or acid protease (0.015% w/v) enzyme solution were added into the mixing vessel (volume 15 L with 300 mm dia. 220 mm height) then, the mixing vessel was tightly closed. The temperature of the mixing vessel was controlled at 10°C. Valve A and B were opened, gaseous CO_2 was fed to replace air in the headspace of the mixing vessel until pressure increased to 2 MPa. Then, valve B was closed and the regulator valve (valve C) was opened, gaseous CO_2 was fed into the mixing vessel at a rate of 2 L/min controlling by valve C. MB-CO_2 were generated by introducing the mixture of enzyme solution and gaseous CO_2 to a swiveling microbubbles generator (56 mm diameter x 86 mm long, outlet and inlet diameter are 5 mm and 13 mm, respectively; BT-50; Bubble tank Co., Japan) by circulating pump at rate 15 L/min. MB-CO_2 was generated for 10 min, and then valves A and C were closed and the circulating pump was stopped. Next, valve D was opened, the enzyme solution saturated with CO_2 was continuously fed to a heating coil (volume 170 ml with 5 mm dia x 850 mm length) using a metering pump at flow rate of about 13-17 L/min. In this study, the enzyme inactivation was performed under the following conditions: the temperatures of the heating coil were 45 or 50°C, and the pressure of the heating coils was 2, 4 or 6 MPa. The treated enzymes were collected from the sampling valve (valve E) at 0, 10, 20 and 30 min and then immediately taken to measure the level of enzyme activity (%).

Enzyme inactivation with thermal treatment

The efficacy of conventional thermal treatment was compared with MB-CO_2. Five milliliters of glucoamylase (0.004% w/v) or acid protease (0.015% w/v) enzyme solution were placed in a test tube and incubated in a water bath. The temperatures of the water bath were varied between 45 and 70°C. The incubation times were 10, 20 and 30 min.

Measurement of glucoamylase activity

The activity of the glucoamylase was measured using a glucoamylase assay kit (Kikkoman Co., Chiba, Japan). The enzyme activity was determined at pH 4. The assay medium contained 250 µl of β-glucosidase and 250 µl of 4-nitrophenyl β-maltoside as substrate. The substrate was incubated at 37°C for 5 min and then 100 µl of enzyme sample was added to the substrate and incubated for 10 min. Next, 1 ml of sodium carbonate ($NaCO_3$) was added to stop the reaction. The activity of the glucoamylase enzyme was determined with a spectrophotometer at the absorbance of 400 nm (Abs_{400}). The blank sample was prepared by adding the stopping reagent to the mixture before the sample of enzyme. The relative activities of glucoamylase were calculated using the following equation (1):

$$\text{Relative residual activity} = \frac{Abs_{400} \text{ of } t_t \times 100}{Abs_{400} \text{ of } t_0} \quad (1)$$

Where t_t is incubation time (min) and t_0 is initial time (0 min)

Measurement of acid protease activity

The acid protease activity was determined at pH 4. One ml of casein solution (2% w/v) of casein and sodium acetate buffer (pH 4) were mixed and incubated at 37°C for 5 min. Then, 500 µl of protease enzyme was added and incubated for 10 min. The reaction was stopped by 3 ml of 0.4 M trichloroacetic acid to stop the reaction. The mixture was incubated at 37°C for 30 min. After that, the mixture was filtrated. One ml of the filtrated mixture was mixed with 1 ml of 20% (v/v) phenol reagent and 5 ml of 0.55 M Na_2CO_3. The mixture was incubated at 37°C for 30 min. Acid protease activity was determined at the absorbance of 660 nm (A_{660}) using spectrophotometer. The blank sample was prepared by adding the stopping reagent to the mixture before the sample of enzyme. The relative activity of protease was calculated as followed equation (2):

$$\text{Relative residual activity (\%):} \quad \frac{Abs_{660} \text{ of } t_t \times 100}{Abs_{660} \text{ of } t_0} \quad (2)$$

Where t_t is incubation time (min) and t_0 is initial time (0 min)

Kinetic analysis

The kinetic data on the inactivation of glucoamylase and acid protease was calculated using a conventional 1st-order kinetic model [16] was shown in equation (3):

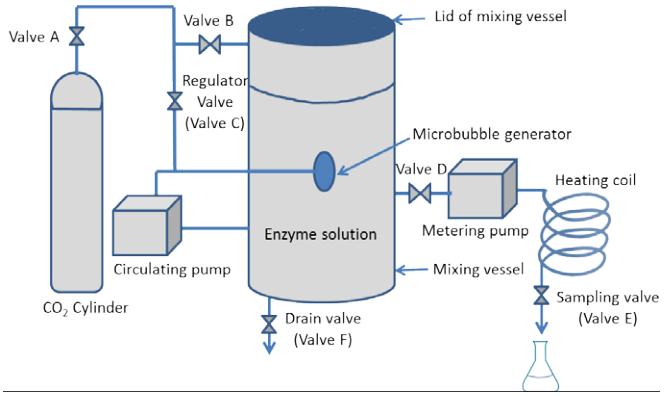

Figure 1: Schematic diagram of carbon dioxide micro bubbles system.

$$\ln(A) = -kt \quad (3)$$

Where A is the residual activity at any residence time (t, min) and k is the reaction rate constant (min^{-1}) at a given condition. The value of k was obtained from the slope of the regression of ln (A) versus time.

The decimal reduction time (D value), defined as the treatment time needed for 90% inactivation of initial activity at a given condition [16] was shown in equation (4):

$$D = 2.303/k \quad (4)$$

The Z value defined as the temperature increment needed for a 90% reduction in the D value at a given condition [16] was shown in equation (5):

$$Z = (T_2-T_1)/ (\log D_1-\log D_2) \quad (5)$$

Where T_1 and T_2 are temperature at a given condition.

Statistical analysis

The data were measured in triplicate. Data were analyzed using the software package SPSS 17.0 for Windows (SPSS Inc., Chicago, IL). Significant differences between mean values were determined using Turkey's honestly significant different (HSD) multiple range test (P=0.05).

Results and Discussion

Table 1 shows the relative activities of glucoamylase at 45 and 50°C with MB-CO_2 treatments in comparison with thermal treatments of 45-70°C. It shows that the inactivation of glucoamylase could not be achieved by temperature lower than 65°C without MB-CO_2. In contrast, the inactivation of glucoamylase was successfully achieved at 45 and 50°C using MB-CO_2. The residual activity of glucoamylase treated by MB-CO_2 at 50°C was lower than that by MB-CO_2 at 45°C. The thermal treatment inactivated glucoamylase by denaturation of enzyme structure. The absorption and co-existence of pressurized CO_2 into the C-terminal affect the decomposition of the α-helix enzyme structure [19,32,33]. The result showed that MB-CO_2 treatment increased the ability of non-thermal treatment on glucoamylase inactivation. The efficacy of MB-CO_2 on enzyme inactivation was increased by increasing the temperature [34] which was possibly caused by increased diffusivity of MB-CO_2 molecules to enzyme structure at higher temperatures. In addition, the efficacy of MB-CO_2 at different pressures in the heating coil on glucoamylase inactivation showed in Figure 2. The residual activity of glucoamylase at 2 MPa was much higher than that at 4 MPa, while glucoamylase activity was slightly decreased when pressure was increased from 4 MPa to 6 MPa (from 39.74% to 36.59%). The correlation coefficient between residual activity of glucoamylase and pressure was – 0.9147. This indicates that the residual activity of glucoamylase was associated with pressure, residual activity of glucoamylase decreases as the pressure increases. In order to save energy, thus, the condition operation at 4 MPa of the heating coil was preferable. The pressure in the heating coil would be responsible for the stability of dissolved MB-CO_2 in enzyme solution [29]. The present data suggests that the pressure of 4 MPa in the heating coil was enough to maintain the stability of dissolved MB-CO_2 when the pressure of mixing vessel was operated at 2 MPa. This result was similar to that reported by Kobayashi et al. [5] who studied on PPO inactivation using MB-CO_2 at 1-4 MPa.

Table 2 shows the rate constants (k), decimal reduction time (D) and Z value. The Z value means as the temperature increase needed for a 90% reduction of D value. The K value of treatments without MB-CO_2 at 45 and 50°C were 0.0093 and 0.0371 min^{-1}, respectively whereas those treated with MB-CO_2 were much higher (0.1493-0.6150 min^{-1}). The lowest D value (3.75 min) was obtained from condition MB-CO_2 at 50°C and 4 MPa whereas the D value of treatment at same temperature (50°C) without MB-CO_2 was up to 177.04 min. The recommended MB-CO_2 condition for glucoamylase inactivation in this study was temperature at 50°C and 4 MPa pressure. Furthermore, Z values of glucoamylase inactivation were 16.05°C and 44.81°C for treatments with and without MB-CO_2 respectively. Lower Z value indicated the lower temperature required. The enzyme inactivation at lower temperature could maintain the sensory quality than at higher temperature.

Table 3 shows the relative activities of acid protease at 45 and 50°C with MB-CO_2 at 4 MPa in comparison with thermal treatments of 45-65°C. The results show that the inactivation of protease by thermal treatments could not be achieved at temperature lower than 65°C. In contrast, the inactivation of acid protease was achieved at 45 and 50°C using MB-CO_2. The residual enzyme activity treated without MB-CO_2 at 50°C was 51.81%, whereas that treated with MB-CO_2 treatment at 50°C was greatly decreased to 1.12%. The residual activity treated using MB-CO_2 at 50°C was similar to that treated using MB-CO_2 at 45°C. Thus, the optimum operating temperature for acid protease inactivation by MB-CO_2 at 45°C was recommended.

Table 4 shows the k, D and Z values of acid protease inactivation. It showed that k value of treatments without MB-CO_2 at 45 and 50°C were 0.0660 min^{-1} and 0.2016 min^{-1}, respectively whereas those treated with MB-CO_2 at 45 and 50°C were higher (1.3866-1.489 min^{-1}). The lowest D value was obtained from MB-CO_2 at 50°C (1.01 min) while a much higher D value (11.46-35.80 min) were obtained from treatment without MB-CO_2 at the same temperature. Z values of acid protease inactivation were 111.24°C and 10.35°C for with and without MB-CO_2, respectively. The high Z value supported that there was no difference

Treatment No.	MB-CO_2	Temperature (°C)	Relative residual activity at 30 min (%)
1	No	45	74.83 ± 1.73[a]
2	No	50	75.03 ± 0.66[a]
3	No	60	65.50 ± 1.95[b]
4	No	65	2.04 ± 0.38[e]
5	No	70	0.37 ± 0.01[f]
6	Yes	45	39.74 ± 0.43[c]
7	Yes	50	15.01 ± 0.96[d]

[a-f] Data in the column with different superscript letters are significantly different (P<0.05).

Table 1: Inactivation of glucoamylase by thermal and MB-CO_2 treatments at 4 MPa.

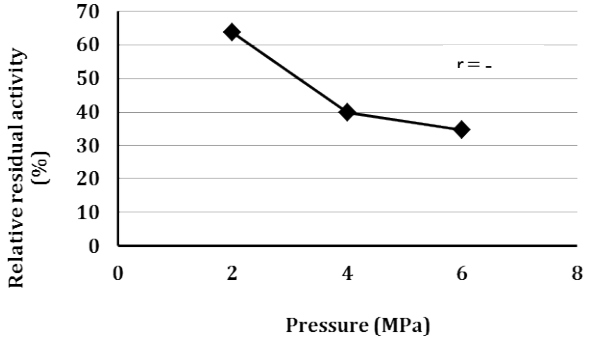

Figure 2: Effect of pressure in a heating coil on relative residual activity of glucoamylase by MB-CO_2 at 45°C.

Treatment	MB-CO_2	Temperture (°C)	Pressure (MPa)	k (min^{-1})	D (min)	Z (°C)
1	No	45	No	0.0093 ± 0.00	248.02 ± 21.21	44.81 ± 14.76
2	No	50	No	0.0371 ± 0.05	177.04 ± 132.04	
6	Yes	45	4	0.2996 ± 0.00	7.69 ± 0.12	16.05 ± 0.92
7	Yes	50	4	0.6150 ± 0.02	3.75 ± 0.11	
8	Yes	45	2	0.1493 ± 0.01	15.48 ± 1.10	
9	Yes	45	6	0.3123 ± 0.01	7.38 ± 0.16	

Table 2: Rate constant (k), decimal reduction time (D) and Z values of glucoamylase inactivation at 45 and 50°C with and without MB-CO_2.

Treatment No.	MB-CO_2	Temperature (°C)	Relative residual activity at 30 min (%)
1	No	45	81.25 ± 3.24^{a}
2	No	50	51.81 ± 1.63^{ab}
3	No	60	35.80 ± 3.03^{bc}
4	No	65	22.01 ± 0.69^{bc}
6	Yes	45	2.29 ± 0.73^{c}
7	Yes	50	1.12 ± 0.45^{c}

[a-c] Data in the column with different superscript letters are significantly different (P<0.05).

Table 3: Inactivation of acid protease by thermal and MB-CO_2 treatments at 4 MPa.

Treatment.	MB-CO_2	Temperature (°C)	Pressure (MPa)	k (min^{-1})	D (min)	Z (°C)
1	No	45	No	0.0660 ± 0.01	35.80 ± 6.80	10.35 ± 1.55
2	No	50	No	0.2016 ± 0.01	11.46 ± 0.75	
6	Yes	45	4	0.3866 ± 0.11	1.67 ± 0.13	111.24 ± 4.36
7	Yes	50	4	1.4289 ± 0.07	1.01 ± 0.08	

Table 4: Rate constant (k) decimal reduction time (D) and Z values of protease inactivation at 45°C and 50°C with and without MB-CO_2.

in acid protease inactivation between MB-CO_2 at 45°C and 50°C. Thus, condition operating for acid protease inactivation at 45°C and 4 MPa of MB-CO_2 treatments was recommended.

Conclusion

In the present study, the efficacy of non-thermal treatment on glucoamylase and acid protease inactivation were increased by addition of MB-CO_2. The inactivation of glucoamylase and acid protease without MB-CO_2 could not be achieved at temperature lower than 65°C. The pressure at 4 MPa in the heating coil was suitable to maintain the stability of dissolved MB-CO_2. The relative residual activity of glucoamylase treated by MB-CO_2 at 50°C was significantly lower than that by MB-CO_2 at 45°C. For acid protease, the relative residual activity treated by MB-CO_2 at 45 and 50°C was not significantly different. The recommended MB-CO_2 condition for glucoamylase and acid protease inactivation in this study were at 50°C and 45°C with 4 MPa, respectively. Accordingly, the present study demonstrated that MB-CO_2 could enhance the efficacy of temperature at 45-50°C on enzyme inactivation.

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