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Effect of CO_2 Flow Rate on Enzyme Inactivation by Continuous Method with Microbubbles of Supercritical Carbon Dioxide

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In our previous papers, batch treatment with microbubbles of supercritical carbon dioxide ($SC–CO_2$) was established as an alternative method to heat treatment. For adapting to food industry, continuous system with microbubbles of $SC–CO_2$ was designed and constructed. In continuous system, there are new factors affecting inactivation efficiency of microbubbles $SC–CO_2$ treatment. In this paper, effect of CO_2 flow rate on enzyme inactivation by continuous method with microbubbles of $SC–CO_2$ was investigated. Dissolved CO_2 concentration, which played an important role for enzyme inactivation by microbubbles $SC–CO_2$ treatment, was enhanced depending on CO_2 flow rate. Also, it was observed that pH of deionized water was lowered to below 3 during the treatment. α–amylase, which was thermoresistant enzyme, was easily inactivated at low CO_2 flow rate because of temporary lowering of pH. On the other hand, inactivation efficiency of acid protease, which was acid–resistant enzyme, was increased depending on CO_2 flow rate.

INTRODUCTION

In food industry, heat treatments generally have been used for inactivation of enzymes and microorganisms. These treatments, however, result in critical damages of the food quality, such as flavor and taste. Therefore, developments of a novel method have been desired, and many researchers have tried to establish alternative methods, such as hydrostatic pressure, microwave heating, pulsed electric field, and so on. Seyderhelm *et al.* (1996) have reported inactivation of pectinesterase, polyphenol oxidase, peroxidase, lipase, lipoxygenase, and so on, using ultra high pressure treatment, but impractical high pressure (higher than 500 MPa) was needed for their significant inactivation. Yen and Lin (1996) have described that inactivation of pectinesterase and polyphenol oxidase was difficult by hydrostatic pressure treatment at 25 °C and 600 MPa for 15 min. Ancos *et al.* (1999) have used microwave heating for inactivation of polyphenol oxidase and peroxidase in strawberry, papaya and kiwi. Although these treatments

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have demonstrated higher inactivation efficiency rather than heat treatment, they are not widely applied to the industrial world because of cost for apparatus or difficulty to design continuous system, and so on.

In recent years, supercritical fluids have been used as environmentally benign media in various fields, such as enzymatic reactions (Kamat *et al.*, 1993; Randolph *et al.*, 1998; Mesiano *et al.*, 1999), extractions (Ibáñez *et al.*, 1999; Simándi *et al.*, 1999), dyeing (Sicardi *et al.*, 1999; Santos *et al.*, 2001), degradation of polymer compounds (Savage, 1999; Kim *et al.*, 2001). In such fluids, supercritical carbon dioxide (SC–CO_2) has several advantages, such as nontoxicity, nonflammable, and no residual chemical problem. Also, SC–CO_2 is fluid that is easy to operate because of moderate critical temperature and pressure (critical temperature; 31.1 °C: critical pressure; 7.4 MPa).

We have developed an alternative method with microbubbles of SC–CO_2 for inactivation of enzymes and microorganisms in liquid product (sake, juices, and so on) under moderate conditions (Ishikawa *et al.*, 1995, 1996a, 1997). In the previous papers, batch system was used for inactivation of enzymes and microorganisms. For improving the treatment efficiency and adapting microbubbles of SC–CO_2 method to food industry, a new apparatus for continuous system with microbubbles SC–CO_2 was designed and constructed. In a batch method, temperature, pressure and time related to microbubbles SC–CO_2 treatment are main operating factor, whereas in a continuous system, there are new factors for affecting inactivation efficiency of microbubbles SC–CO_2 treatment, such as CO_2 flow rate. Our previous papers have reported that inactivation of enzymes and microorganisms by batch method with microbubbles of SC–CO_2 was predominantly affected to dissolved CO_2 concentration during the treatment. In this paper, we tried to evaluate dissolved CO_2 concentration during continuous method with microbubbles of SC–CO_2, and elucidate the effect of CO_2 flow rate on enzyme inactivation by continuous method with microbubbles of SC–CO_2.

MATERIALS AND METHODS

Continuous treatment with microbubbles of SC–CO_2

For continuous treatment with microbubbles of SC–CO_2, an instrument was manufactured by Shimadzu Co. (Kyoto, Japan) according to our design (Figure 1). Sample and liquid CO_2 were simultaneously pumped through a treatment vessel (2.8 cm i.d.×35 cm long, 215 mL of inner volume) in respective flow rates with plunger–type variable speed compressors. Liquid CO_2 changed to gaseous or supercritical state when came into evaporator before treatment vessel, and then was supplied to treatment vessel through a micropore filter (10 μm of pore size). Treatment time is defined as average residence time (RT) calculated from sample flow rate and inner volume of treatment vessel.

Measurement of CO_2 concentration in deionized water during continuous treatment with microbubbles of SC–CO_2

Dissolved CO_2 concentration in deionized water during continuous treatment with microbubbles of SC–CO_2 was measured using dry gas meter. After depressurization to atmospheric pressure, CO_2 that could not dissolve in deionized water was separated from the solution because excess CO_2 dissolved in deionized water during microbubbles

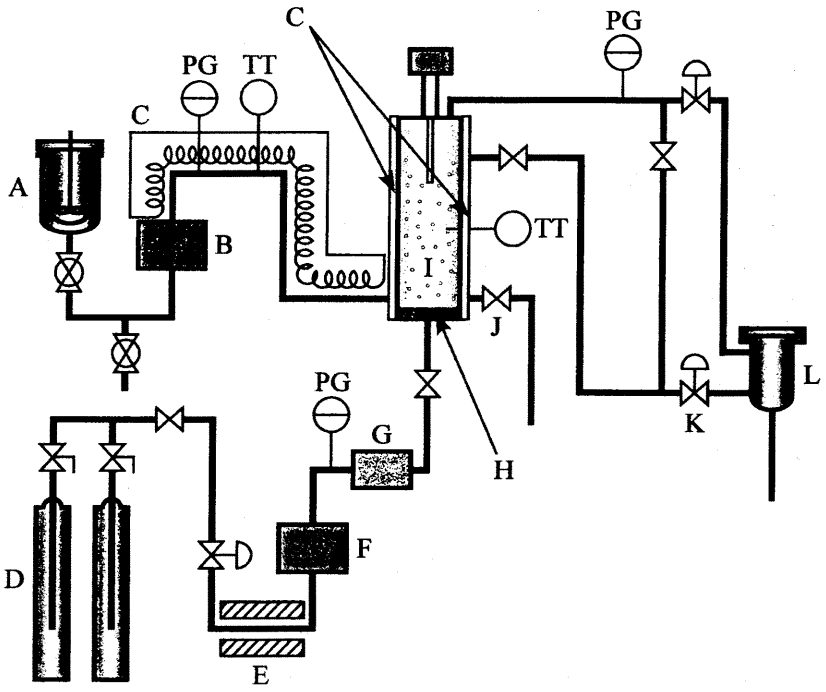

Fig. 1. Schematic of apparatus for continuous treatment with microbubbles of SC–CO_2. Symbols indicate: A, sample vessel; B, sample pump; C, heater; D, CO_2 cylinder; E, cooling unit; F, CO_2 pump; G, evaporator; H, micropore filter (10 μm of pore size); I, treatment vessel (215 mL of inner volume); J, drain valve; K, pressure–regulating valve; L, product vessel; PG, pressure gauge; TT, thermoelectric thermometer.

SC–CO_2 treatment. Treated solution and separated CO_2 passed through a trap for dividing CO_2 from treated solution. Then, the volume of separated CO_2 was measured with dry gas meter. The CO_2 concentration was calculated as Kuenen coefficient. Kuenen coefficient is defined as a volume (mL) of gas at 0 °C and 760 mmHg calculated from a volume of gas dissolved into 1 g of a solvent at arbitrary temperature and pressure.

Observation of pH change in treatment vessel during continuous treatment with microbubbles of SC–CO_2

A treatment vessel equipped with a pressure–resistant window was used for visual observation of pH change during continuous treatment with microbubbles of SC–CO_2. Bromophenol blue (BPB) and thymol blue as pH indicators were dissolved in water, and then subjected to continous treatment with microbubbles of SC–CO_2. During treatment, the change of pH in the treatment vessel was photographically observed.

Preparation of enzyme solutions

α–Amylase from *Bacillus subtilis* (EC.3.2.1.1, Amano Enzyme Inc., Nagoya, Japan)

and acid protease from *Aspergillus niger* (EC.3.4.23.6, Hankyu Bioindustry Co., Ltd., Osaka, Japan) were dissolved in deionized water in a concentration of 0.1 mg mL^{-1}. And then each enzyme solution was subjected to continuous treatments with microbubbles of SC–CO_2 at arbitrary conditions.

Measurements of enzyme activity

α–Amylase; The activity of α–amylase was measured by using amylase assay kit (amylase B–test Wako from Wako Pure Chemical Industry, Osaka, Japan). One unit of the enzyme activity is defined as the amount of enzyme required to degrade fully 10 mg of starch at 37 °C for 30 min.

Acid protease; the activity of acid protease was determined by the method of Ishikawa *et al.* (1995). One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μg of tyrosine per 60 min under the assay conditions.

Residual activity

The residual activity was defined as a percentage of the activity (units mL^{-1}) of enzyme solution after continuous treatment with microbubbles of SC–CO_2 against the activity (units mL^{-1}) of enzyme solution prepared.

RESULTS AND DISCUSSION

Figure 2 indicates behavior of dissolved CO_2 concentration in deionized water during continuous treatment with microbubbles of SC–CO_2. Continuous treatments with

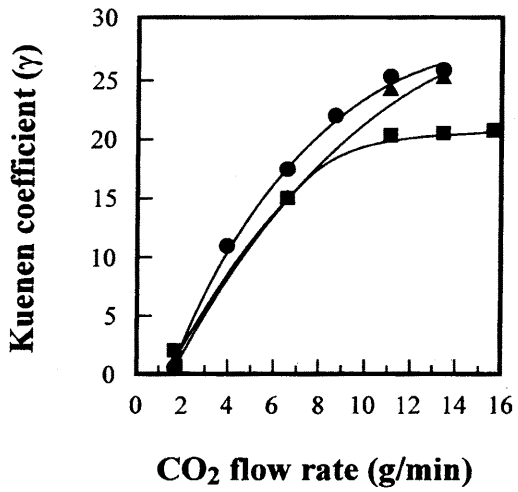

Fig. 2. Effect of CO_2 flow rate on dissolved CO_2 concentration during continuous treatment with microbubbles of SC–CO_2. Microbubbles SC–CO_2 treatment was carried out at 35 °C (●), 40 °C (▲), 45 °C (■), 10 MPa and 15.6 g–CO_2/min for 5 min of RT.

microbubbles of SC–CO_2 were carried out at 35, 40, 45 °C, and 10 MPa for 5 min of RT. The dissolved CO_2 concentration was enhanced depending on CO_2 flow rate. At 10 MPa and 13.4 g–CO_2/min, Kuenen coefficient was 26.1 at 35 °C, 25.3 at 40 °C and 20.7 at 45 °C. The saturated concentrations at 10 MPa and each temperature were 29.1 at 35 °C, 27.8 at 40 °C and 26.5 at 45 °C, respectively. Irrespective of the changes in sample flow rates and treatment pressure, dissolved CO_2 concentration enhanced similarly (data not shown). In batch system, Ishikawa *et al.* (1995) have reported that increase of dissolved CO_2 concentration in sample solution played an important for enzyme inactivation because higher inactivation effect was obtained with higher dissolved CO_2 concentration. Therefore, this continuous system with microbubbles of SC–CO_2 might be able to inactivate enzymes regardless of treatment conditions.

Arreora *et al.* (1991) and Balaban *et al.* (1991) have described that enzyme inactivation by SC–CO_2 treatment was based on the temporary lowering of pH during treatment because dissolution of high-pressure CO_2 molecules to water resulted in production of carbonic acid. Then, using BPB and thymol blue as pH indicators, the change of pH during microbubbles SC–CO_2 treatment was visually observed (Figure 3). Continuous treatment with microbubbles of SC–CO_2 was carried out at 35 °C, 20 MPa, and

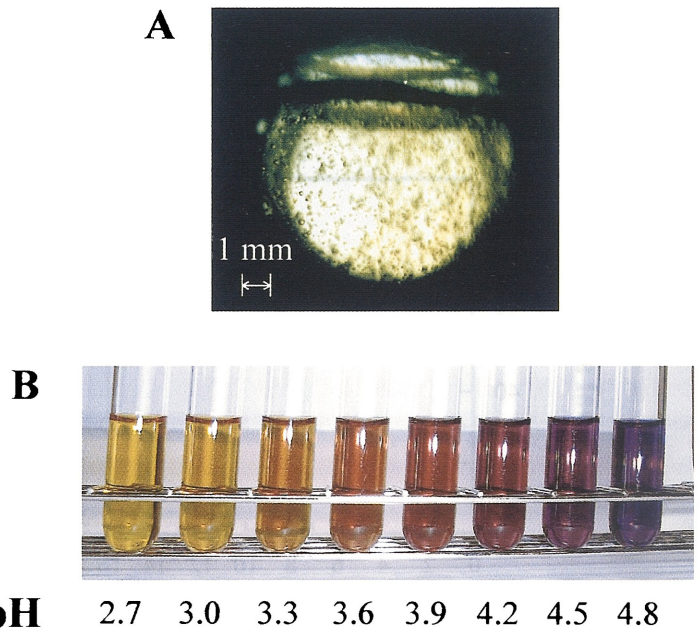

Fig. 3. pH change of deionized water induced by continuous treatment with microbubbles of SC–CO_2 (A). Microbubbles SC–CO_2 treatment was carried out at 35 °C, 20 MPa and 15.6 g –CO_2/min for 5 min of RT. Photograph of B shows color difference of BPB at each pH value in McIlvaine buffer.

15.6 g-CO_2/min. Before the treatment, BPB solution indicated blue (higher than pH 4.6), and then, the color of the treating solution turned to yellow (less than pH 3.0). After the treatment, BPB solution returned to blue. On the other hand, the color of thymol blue, which indicates from pH 1.2 to pH 2.8, did not change before and during the treatment, that is, the pH during the treatment was above 2.8 (data not shown). Toews *et al.* (1995) have reported that the pH of water in contact with CO_2 at subcritical and supercritical state ranged between 2.8 and 2.95 at 25–70 °C and 70–200 atm. This report agrees with our results, hence temporary lowering of pH was occurred during continuous treatment with microbubbles of SC–CO_2.

The effect of CO_2 flow rate on inactivation of α-amylase by continuous treatment with microbubbles of SC–CO_2 is shown in Figure 4. Continuous treatment with microbubbles of SC–CO_2 was carried out at 35 °C, 30 MPa for 15 min of RT. α-amylase is heat-stable enzyme. After heat treatment at 70 °C for 15 min, residual activity of this enzyme decreased to 97.9%. On the other hand, after microbubbles SC–CO_2 treatment, α-amylase was completely inactivated in low CO_2 flow rate (4.0 g-CO_2/min). When α-amylase dissolved in McIlvaine buffer (pH 3.0) was treated with heat at 35 °C for 15 min, the residual activity was 1.0% (data not shown). Optimum pH of α-amylase is 5.8–6.8, therefore it was considered that inactivation of α-amylase by continuous treatment with microbubbles of SC–CO_2 was mainly attributed to lowering of pH during the treatment in spite of lower CO_2 flow rate.

Figure 5 illustrates effect of CO_2 flow rate on inactivation of acid protease by continuous treatment with microbubbles of SC–CO_2. Continuous treatments with microbub-

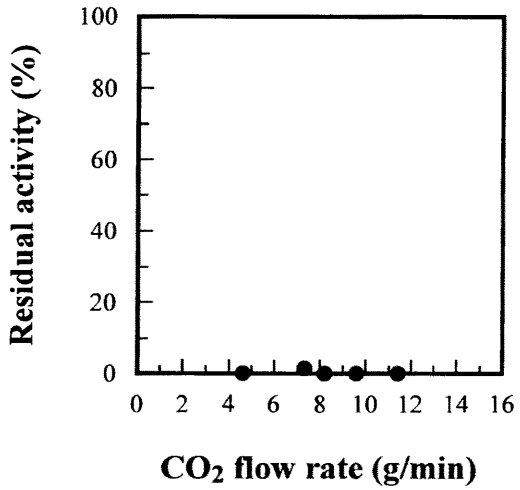

Fig. 4. Effect of CO_2 flow rate on inactivation of α-amylase by continuous treatment with microbubbles of SC–CO_2. Microbubbles SC–CO_2 treatment was carried out at 30 MPa and 15.6 g-CO_2/min for 15 min of RT. Symbol indicates: ●, 35 °C.

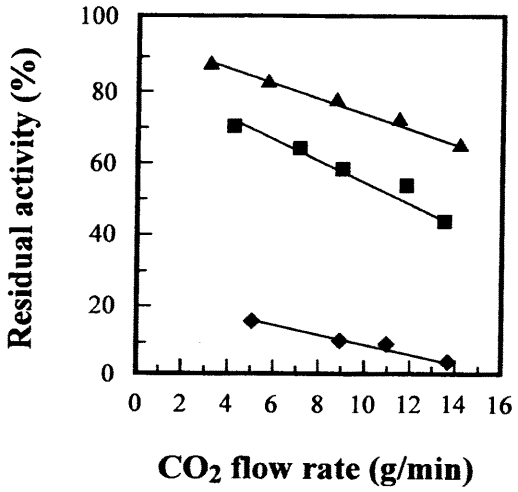

Fig. 5. Effect of CO_2 flow rate on inactivation of acid protease by continuous treatment with microbubbles of SC-CO_2. Microbubbles SC-CO_2 treatments were carried out at 30 MPa and 15.6 g-CO_2/min for 15 min of RT. Symbols indicate:▲, 40 °C;■, 45 °C;●, 50 °C.

bles of SC–CO_2 were carried out at 40 °C, 45 °C, 50 °C, and 30 MPa for 15 min of RT. After heat treatment at 50 °C for 15 min, residual activity of acid protease was 88.6% in deionized water and 90.6% in McIlvaine buffer (pH 3.0). After microbubbles SC–CO_2 treatment, residual activity decreased in proportion to CO_2 flow rate at each temperature. Residual activities after microbubbles SC–CO_2 treatment decreased from 85.5% (3.2 g–CO_2/min) to 63.0% (14.2 g–CO_2/min) at 40 °C, from 68.5% (4.2 g–CO_2/min) to 42.2% (13.5 g–CO_2/min) at 45 °C, and 14.9% (5.1 g–CO_2/min) to 3.2% (13.7 g–CO_2/min) at 50 °C. Ishikawa *et al.* (1996b) have described that inactivation effects were mainly attributed to pH–lowering effect and subsequently conformational change was caused by CO_2 molecules, that is irreversible destruction of α-helix. Kamat *et al.* (1995a, 1995b) have reported that the carbamate complexes were formed between amino groups on enzyme surface and CO_2 molecules. From these results it was considered that temporary lowering of pH induced by dissolution of CO_2 could influence conformation of enzyme, and then the contact of CO_2 molecules with amino groups on the enzyme surface was enhanced by the increase of CO_2 flow rate, as a result, enzymes were inactivated.

These results lead to the conclusion that CO_2 flow rate plays a significant role to enhance not only dissolved CO_2 concentration but also inactivation efficiency. Therefore, it was suggested that continuous treatment with microbubbles of SC–CO_2 might be inactivating various enzymes more easily.

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