pH-Dependent Inactivation of Enzymes by Microbubbling of Supercritical Carbon Dioxide

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The effect of pH on the inactivation of some enzymes (acid protease, alkaline protease, papain, and glucoamylase) dissolved in McIlvaine buffer by microbubble supercritical (SC) CO2 treatment was investigated in the range of pH 3 to pH 6. Remarkable inactivation was observed with this treatment at pH less than 4, whereas with gaseous and liquid CO2 treatments little or no important inactivation occurred. Microbubbling of SC-CO2 at lower pH was effective at 40-50°C. Microbubbling of SC-CO2 at pH 3 inactivated completely enzymes at temperatures 25°C lower than that of the thermal treatment (65-75°C). The degree of inactivation in acid protease increased with increasing ethanol concentration.

Keywords: supercritical, CO2, microbubbles, inactivation, enzyme

The action of enzymes and microorganisms in food products causes deterioration of quality and bacterial spoilage during storage or distribution. Thermal treatment, however, results in loss of heat-sensitive nutrients, denaturation of proteins and changes in texture, color, and flavor, etc. The development of alternative techniques has been desired in the food industry.

Supercritical carbon dioxide (SC-CO2) has been used extensively for extractions (Vega et al., 1996), chromatography (Ibáñez et al., 1994), and modifying chemical reaction (Jessop et al., 1994) because of its moderate operational conditions (low critical temperature and pressure). Carbon dioxide is also nontoxic, nonflammable, inexpensive, and readily available. In recent years, SC-CO2 and/or subcritical CO2 has been applied to inactivate enzymes which are concerned in the degradation of food quality. Pectinesterase in orange juice has been partially inactivated by SC-CO2 at 40–60°C and 31 MPa (Balaban et al., 1991). Chen et al. (1992) investigated the inactivation of polyphenol oxidases from lobster, brown shrimp, and potato by subcritical CO2. Inactivation of oxidoreductases containing metals by SC-CO2 was reported by Endo et al. (1995). In our previous papers (Ishikawa et al., 1995a,b, 1996a,b), the microbubbles of SC-CO2 treatment was proposed as a novel method for enzyme inactivation. Some enzymes which could be responsible for degrading food quality were significantly inactivated at 25 MPa and 35°C for 30 min (Ishikawa et al., 1995a). ‘Sake’ treated with microbubbles of SC-CO2 had preserved its sensory quality close to that of ‘Namazake’ (non heat-treated sake), while the heat-treated sake lost its freshness (Ishikawa et al., 1995b). The conformational changes in enzyme molecules by microbubbling of SC-CO2 were investigated by circular dichroism (CD) spectra analysis (Ishikawa et al., 1996b). A close relationship was observed between the loss of activity and the decomposition of the α-helix structure.

The enzyme inactivation with SC-CO2 would be influenced by the environmental factor, pH because the stability of enzyme molecules in an aqueous solution is pH sensitive (Kamat et al., 1995). The effect of pH on the enzyme stability under the SC-CO2 conditions has been poorly understood. The object of the present investigation was to clarify the pH dependence of the enzyme inactivation by the microbubble SC-CO2 treatment, and by the gaseous and liquid CO2 treatments. The effect of ethanol concentration on enzyme inactivation at the experimental pH was also investigated.

Materials and Methods

Enzyme solutions Acid protease from Aspergillus niger (E.C. 3.4.23.6), alkaline protease from Bacillus subtilis (E.C. 3.4.21.14), and glucoamylase from Rhizopus delemar (E.C. 3.2.1.3) were purchased from Nagase Seikagaku Kogyo Co., Ltd. (Kyoto). Papain (E.C. 3.4.22.4) from Carica papaya was purchased from Merck (Darmstadt, Germany). These en-zymes were considered to be responsible for degrading food quality, so their inactivation in deionized water was investigated in our previous paper (Ishikawa et al., 1995a).

McIlvaine buffer was selected in our experiment because of its wide buffering range. To investigate the effect of the ethanol concentration on the SC-CO2 treatment, ethanol was added to McIlvaine buffer at various concentrations [2.5, 5.0, 7.5, 10, 15% (v/v)]. Each enzyme preparation was dissolved in the buffer solution at the concentration of 50–100 mg/ml.

Apparatus and procedure for microbubbling liquid and SC-CO2 The liquid and SC-CO2 treatments were carried out using a Milton Roy X-10 system (Riviera Beach, FL). This system consists of a 120-ml stainless steel vessel which has a cylindrical
filter (10-µm pore size) made of sintered stainless steel for microbubbling liquid and SC-CO₂ (Ishikawa et al., 1995a). A thermocouple was placed inside the treatment vessel to control temperature. The system had a backpressure regulating valve that could be used to control pressure.

For each experimental trial, 100-ml of enzyme solution was loaded into the treatment vessel, and then pressurized with CO₂. The feeding rate of CO₂ was set at 4.0 g/min. The feeding of CO₂ was continued during the treatment, and the temperature and pressure were kept constant. The liquid- and SC-CO₂ treatments were carried out at 25°C and 35–65°C, respectively, at 30 MPa for 30 min. After each treatment, the vessel was slowly depressurized by releasing CO₂ over a period of about 5 min.

Procedure for microbubbling gaseous CO₂. The gaseous CO₂ treatment was carried out using a 200-ml glass vessel. The gaseous CO₂ was fed into a solution using a filter (10-µm pore size) similar to that used for microbubbling of SC-CO₂. The enzyme solution (100 ml) was kept in a water bath while gaseous CO₂ was bubbled under atmospheric pressure and then cooled rapidly in ice water. The change in pH during the gaseous CO₂ treatment was monitored using a pH meter.

Procedure for thermal treatment. The thermal treatment (temperature control) was carried out using a 200-ml glass vessel. The enzyme solution (100 ml) was kept in a water bath at each experimental temperature under atmospheric pressure, and then cooled rapidly in ice water.

Measurement of enzymatic activity

Proteases assay. The acid protease, alkaline protease, and papain solutions were adjusted to pH at 3.0, 8.0, and 8.0, respectively, and then each was subjected to an assay of enzymatic activity. Proteolytic activity was determined at 37°C with 2% casein as a substrate (Ishikawa et al., 1995a). One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μg of tyrosine per min under the assay conditions.

Glucosamylase assay. The enzymatic activity was determined at pH 5.0 with 2% starch as a substrate, as described previously (Ishikawa et al., 1995a). One unit of enzyme activity is defined as the amount required to liberate 1 mg of glucose per min under the assay conditions.

Results and Discussion

Changes in pH of McIlvaine buffer by the microbubble gaseous, liquid, and SC-CO₂ treatments. To investigate the effect of pH on the enzyme inactivation, it is necessary to consider lowering the pH of the buffer by microbubbling of gaseous, liquid and SC-CO₂. Table 1 shows the pH in McIlvaine buffer (pH 3.0–7.0) after the microbubbling. The microbubbles gaseous CO₂ treatment was carried out at 25°C under atmospheric pressure for 30 min. No important change in pH was observed on the buffer at pH 3.0–6.0 after this treatment. By microbubbling of gaseous CO₂, the pH was steadly lowered to 6.5 within 5 min, and remained constant during the treatment for 30 min. The lowered pH was restored to 6.8 after 6 h (data not shown). The pH lowering effects were also observed on the microbubbles liquid and SC-CO₂ treatments; these were carried out at 25 and 40°C, respectively, and 30 MPa for 30 min. Direct pH measurement was not performed in our experiments because the pH probe for the high pressure process was not available. The pH of the buffer dropped from 7.0 to 6.5 after both treatments. Changes in the buffer at pH 6.0 were less than 0.1, and no important changes were observed at pH 3.0–5.0.

The pH lowering by microbubbling of gaseous, liquid, and SC-CO₂ is attributable to the dissociation of carbonic acid formed by the dissolution of CO₂ into the McIlvaine buffer. The first dissociation constant of carbonic acid at 25–250°C and 1–200 MPa was reported by Read (1975). Under our experimental conditions, the pK values of carbonic acid would be ranged from

![Table 1. Changes in pH of McIlvaine buffers after the microbubbling of gaseous, liquid, and SC-CO₂ for 30 min.](image)

**Table 1. Changes in pH of McIlvaine buffers after the microbubbling of gaseous, liquid, and SC-CO₂ for 30 min.**

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**Means and standard deviations of triplicate measurements.**
6.1 to 6.4. The pH of the buffer tended to be lowered by the dissolution of CO$_2$ at higher pH than the pK value of carbonic acid under these conditions.

Comparison of the inactivation of acid protease by the microbubble gaseous, liquid, and SC-CO$_2$ treatments The effect of pH on enzyme inactivation by microbubbling of gaseous, liquid and SC-CO$_2$ was observed at pH 3–6 using McIlvaine buffer. Figure 1 shows the inactivation of acid protease at pH 3–6 after these treatments. The gaseous CO$_2$ treatments were carried out at 25 and 40°C under atmospheric pressure for 30 min. The liquid and SC-CO$_2$ treatments were carried out at 25 and 40°C, respectively, and submitted to 30 MPa for 30 min. The most remarkable inactivation was observed on the microbubbles SC-CO$_2$ treatment at pH less than 4. The activity of acid protease decreased from 51 to 12 units/ml by microbubbling SC-CO$_2$ at pH 3. A similar inactivation behavior on the SC-CO$_2$ treatment was observed using 0.1 M citrate buffer at pH 3–5 (data not shown). Microbubbling of liquid CO$_2$ also caused it to lose the activity at acidic pH, though the degree of inactivation was considerably smaller than that of SC-CO$_2$. On the other hand, no important loss in activity was observed on the microbubbles gaseous CO$_2$ treatments. The results suggest that acid protease activity was not lost at the water-gaseous CO$_2$ interface under our experimental conditions.

Inactivation of alkaline protease, glucoamylase, and papain by the microbubble SC-CO$_2$ treatment The inactivation of alkaline protease, glucoamylase, and papain by microbubbling SC-CO$_2$ was investigated over the range of pH 3–6. Figure 2 shows the inactivation of the enzymes in McIlvaine buffer after treatment at 40°C and 30 MPa for 30 min. Alkaline protease was inactivated completely under these experimental pH conditions, due to the liability of alkaline protease at this pH range. Moreover, the pH-dependent inactivation by microbubbling SC-CO$_2$ was also observed in the treatments of glucoamylase and papain. The degree of inactivation increased as pH became lower. At pH 3, glucoamylase was completely inactivated and the activity of papain decreased from 72.5 to 14.5 units/ml.

Microbubbling of SC-CO$_2$ causes the decomposition of the α-helix structure in the enzyme molecules in an aqueous solution, which results in loss of activity (Ishikawa et al., 1996b). The stability in the α-helix structure decreased in acid solution, and consequently, the protein molecule was readily subjected to the conformational change (Johnson, 1988). Therefore, the microbubbles SC-CO$_2$ treatment would cause a considerable decomposition of the α-helix structure in enzyme molecules in an acid environment.

Comparison of the microbubble SC-CO$_2$ and the thermal inactivations The inactivations of glucoamylase, papain, and acid protease by the microbubbles SC-CO$_2$ treatment were compared with those by the thermal treatment. The inactivation curves at pH 3 and 5 are shown in Fig. 3 and 4, respectively. At pH 3, the microbubbles SC-CO$_2$ treatment caused complete inactivation at a temperature 25°C lower than that of the thermal treatment. Even with the SC-CO$_2$ treatment at 35°C, glucoamylase was inactivated to 9.2 units/ml. Papain and acid protease were strongly inactivated by microbubbling of SC-CO$_2$ above 40°C. At pH 5, the inactivation curve on the SC-CO$_2$ treatment is drawn at the temperature 15°C higher than the curve obtained at pH 3. In this instance, the temperature difference between the SC-CO$_2$ and thermal treatments, when complete inactivation occurred, was smaller than that observed at pH 3. The complete inactivation of acid protease at pH 5 was observed at a temperature only 5°C less than that of the thermal treatment (70°C). The results suggest that microbubbling of SC-CO$_2$ at lower pH would also be effective at lower temperature. Thus, enzymes in food products which have acidic pH would be efficiently inactivated. For instance, orange juice (pH 3.4–3.7) has been treated by
pH-Dependent Enzyme Inactivation by SC-CO<sub>2</sub>. As shown in Fig. 4, a high temperature (35°C and 25 MPa for 30 min) easily inactivated at 35°C and 25 MPa for 30 min. Pectinesterase, which has a high thermostability in orange juice, has been inactivated at pH 5 using the microbubble SC-CO<sub>2</sub> treatment. In contrast, acid protease in sake (pH 4.4) was readily inactivated at 35°C (Ishikawa et al., 1996a). Pectinesterase in orange juice is inactivated at 35°C and 30 MPa for 30 min. At 25°C, the degree of inactivation increased with increase in the ethanol concentration. Figure 5 shows the effect of ethanol concentration on the inactivation of acid protease at pH 3 and 5 by microbubbling of SC-CO<sub>2</sub>. The pronounced difference of these two inactivation temperatures is chiefly attributable to the ethanol concentration. Figure 5 shows the effect of ethanol concentration on the inactivation of acid protease at pH 3 and 5 by microbubbling of SC-CO<sub>2</sub> at 35°C and 30 MPa for 30 min. At pH 5, acid protease was inactivated to 2.4 units/ml in 15% (v/v) ethanol solution, while its activity was 75% at pH 3 in 5% (v/v) ethanol solution. These results indicate that the microbubble SC-CO<sub>2</sub> treatment would be effective for alcoholic beverages such as beer, sake, and wine.

A higher concentration of ethanol would result in a decrease in dielectric constant in the solution, and a decrease in the intramolecular electrostatic interactions inside the enzyme molecules. In consequence, the conformation of the enzyme molecule would be unstable in ethanol solution, and easily changed by microbubbling of SC-CO<sub>2</sub>.

References