Inactivation of Enzymes and Decomposition of α-Helix Structure by Supercritical Carbon Dioxide Microbubble Method

Hiroya Ishikawa,* Mitsuya Shimoda, Akiyoshi Yonekura, and Yutaka Osajima

Department of Food Science and Technology, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812, Japan

The conformational changes of enzymes inactivated by the supercritical CO_2 (SC CO_2) microbubble method were investigated by measuring the circular dichroic (CD) spectra at far ultraviolet (UV) range. The decay of negative ellipticity of lipase, alkaline protease, acid protease, and glucoamylase was observed after the SC CO_2 treatment at 35 °C and 25 MPa for 30 min. The residual α-helix contents of these enzymes were 62.9, 31.3, 37.6, and 12.4%, respectively. On the other hand, the α-helix structure of glucoamylase remained 85.7% after the heat treatment at 80 °C for 30 min. Furthermore, residual α-helix content of glucoamylase treated with SC CO_2 decreased from 12.4 to 5.1% during 10 days at 20 °C, and the residual enzyme activity also decreased from 17.5 to 0%. The plots of the residual activities against the residual α-helix contents gave a linear relationship both for glucoamylase and acid protease.

Keywords: *Supercritical CO_2; microbubble; inactivate; conformational change*

INTRODUCTION

The action of enzymes and microorganisms in various foods cause deterioration of quality and bacterial spoilage during storage or distribution. Heat treatment is widely used to prevent these biochemical changes. This treatment, however, is often accompanied with undesirable changes in the quality of food. The development of alternative techniques to prevent undesirable changes during heat treatment is desired in the food industry.

Recent reports have dealt with the influence of supercritical carbon dioxide (SC CO_2) on enzymes. Taniguchi et al. (1987) investigated the retention of activity of α-amylase, glucose oxidase, lipase, and catalase with water content of 5−7 wt% under SC CO_2 conditions. Balaban et al. (1991) investigated the inactivation of pectinesterase in orange juice by SC CO_2. Arreola et al. (1991) reported the effects of SC CO_2 on some quality attributes of orange juice. However, this SC CO_2 treatment seems to be lacking efficiency because it is necessary to prolong the length of treatment to get sufficient effects.

As a novel method for the inactivation of enzymes and the sterilization of microorganisms, the SC CO_2 microbubble method was proposed in our previous papers (Ishikawa et al., 1995a−d). This SC CO_2 treatment (SCT) method, in which microbubbles of SC CO_2 are put into aqueous solution through a filter with a 10-μm pore size, is highly efficient at inactivating enzymes and sterilizing microorganisms. With this SCT method, enzymes in aqueous solution were effectively inactiated at 25 MPa and 35 °C for 30 min (Ishikawa et al., 1995a). *Lactobacillus brevis* and *Saccharomyces cerevisiae* were completely sterilized by the SC CO_2 treatment at 25 MPa and 35 °C for 30 min (Ishikawa et al., 1995c). The residual activity of pectinesterase in orange juice decreased to 5% after treatment at 25 MPa and 35 °C for 30 min (Ishikawa et al., 1995d). Sake treated by microbubbling of SC CO_2 at 20 MPa and 35 °C for 30 min preserved the sensory quality near to that of Namazake, whereas heat-treated Sake lost its freshness (Ishikawa et al., 1995b).

In our previous study (Ishikawa et al., 1995a), we considered that factors other than pH, temperature, pressure, and surface tension at the CO_2−water interface must have contributed to the enzyme inactivation. Thus, we presumed that the sorption of SC CO_2 into the enzyme molecule would cause the conformational changes and then result in the loss of activity. The purpose of this paper was to confirm the conformational change of the enzyme molecule by the SC CO_2 microbubble method and clarify the relationship between the loss of activity and the conformational change.

MATERIALS AND METHODS

Enzyme Solutions. Acid protease from *Aspergillus niger* (EC 3.4.23.6), alkaline protease from *Bacillus subtilis* (EC 3.4.21.14), glucoamylase from *Rhizopus delemar* (EC 3.2.1.3), and lipase from *Rhizopus japonicus* (EC 3.1.1.3), each of which could be responsible for degrading food quality, were purchased from Nagase Seikagaku Kogyo Company Ltd. These enzymes were dissolved in deionized water to a concentration of 10 μg/mL for the SC CO_2 microbubble method (SCT) and/or spectropolarimetric analysis.

Apparatus and Procedure for SCT. SCT was performed with a Milton Roy X-10 system (Riviera Beach, FL). This system consisted of a 120-mL treatment vessel that has a cylindrical filter made of sintered stainless steel for feeding SC CO_2 in microbubbles (Ishikawa et al., 1995a).

For each experiment, 100 mL of enzyme solution was loaded into the treatment vessel, which had been preheated to the experimental temperature, and the pressurized by CO_2. The temperature and pressure were maintained at experimental levels. The treatment conditions were set similar to the conditions in our previous paper (Ishikawa et al., 1995a); that is, the pressure ranged from 8 to 30 MPa and the temperature ranged from 35 to 50 °C. The CO_2 was fed at 4.0 g/min about ∼10 min until the pressure reached the experimental level, and then feeding was stopped. At the end of the SCT, the vessel was slowly depressurized by releasing the pressure over a period of ∼5 min.

Measurement of Enzymic Activity. *Acid Protease and Alkaline Protease Assay.* The proteolytic activities of acid and alkaline proteases were determined at pH 3.0 and 8.0, respectively, with 2% casein as a substrate, as described

* Author to whom correspondence should be addressed [fax (092) 642-3030].

S0021-8561(96)00207-5 CCC: $12.00

previously (Ishikawa et al., 1995a). 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.

Glucoamylase 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 of enzyme required to liberate 1 mg of glucose per 60 min under the assay conditions.

Lipase Assay. The enzymatic activity was determined at pH 7.0 with olive oil as a substrate, as described previously (Ishikawa et al., 1995a). One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of oleic acid per 60 min under the assay conditions.

Residual Activity. The residual activity of the enzymes was calculated with the following formula:

$$\text{residual activity (\%)} = \frac{\text{activity (unit/mL) of the SCT enzyme solution}}{\text{activity (unit/mL) of the enzyme solution prepared}} \times 100$$

Spectropolarimetric Analysis of Enzymes. Circular dichroic (CD) spectra were scanned at the far UV range (250−200 nm) with a Jasco J-720 spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan) and a Suprasil (Helma Cells) cuvette with 1.0-cm light path. The CD spectra were made at 20 °C. α-Helix content was calculated with the following formula proposed by Greenfield and Fasman (1969):

$$\alpha\text{-helix (\%)} = \frac{[\theta]_{208\ nm} - 4000}{33000 - 4000}$$

Residual α-Helix Content. The residual α-helix content of the enzyme after SCT was calculated with the following formula:

$$\text{residual α-helix content (\%)} = \frac{\text{α-helix content (\%) of SC } CO_2\text{-treated enzyme}}{\text{α-helix content (\%) of untreated enzyme}} \times 100$$

RESULTS AND DISCUSSION

Effect of SC CO_2 on the Secondary Structure of Enzymes. The CD spectra of enzymes (lipase, alkaline protease, acid protease and glucoamylase) at far-UV range are shown in Figure 1. The enzymes were either untreated or treated with SC CO_2 by the microbubble method at 35 °C and 25 MPa for 30 min. After the treatment, the residual activities of lipase, alkaline protease, acid protease, and glucoamylase were 0, 0, 25.3, and 17.5%, respectively (Ishikawa et al., 1995a).

A decay of negative ellipticity of all enzymes was observed after the SCT. The residual α-helix contents of lipase, alkaline protease, acid protease, and glucoamylase were 62.9, 31.3, 37.6, and 12.4%, respectively. These results suggest that the decreases of activity could be related to the decomposition of secondary structures in enzyme molecules caused by SCT. The CD spectra of SC CO_2-treated enzymes were distinct from that of a protein rich in a random coil structure (Greenfield and Fasman, 1969), although the α-helix structure was significantly broken as just described. The α-helix structure undergoes a conformational change in acidic solution, but the β-sheet tends to be more stable (Johnson, 1988). Thus, the α-helix structures could decompose in a carbonated aqueous solution containing microbubbles of SC CO_2. In SCT, the decomposition of α-helix structure would result in the inactivation of enzyme.

Effect of the Filter on Efficiency of SCT. The CD spectra of glucoamylase after the SCT at 25 MPa and 35 °C with or without the micropore filter are shown in Figure 2. The pore size of the filter was 10 μm. The increase of the decay of ellipticity was accelerated by SCT with the filter compared with the treatment without filter; that is, the residual α-helix content decreased from 50.8 to 12.4%. In our previous paper (Ishikawa et al., 1995a), we noted that microbubbling of SC CO_2 increased the CO_2 concentration in aqueous solution and could increase the amount of CO_2 sorbed into enzyme. Thus, it is possible that the increase of the CO_2 sorbed accelerated the decomposition of α-helix structure.

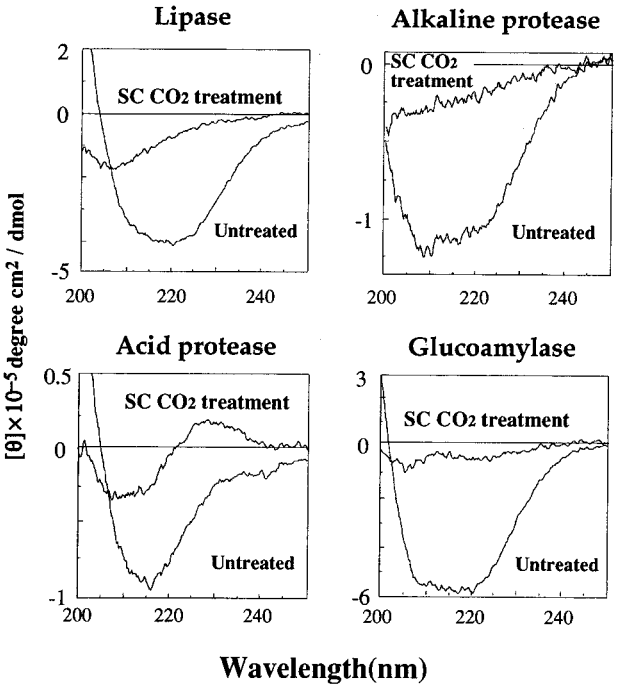

Figure 1. Circular dichroic spectra of lipase, alkaline protease, acid protease, and glucoamylase after SCT at 25 MPa and 35 °C for 30 min.

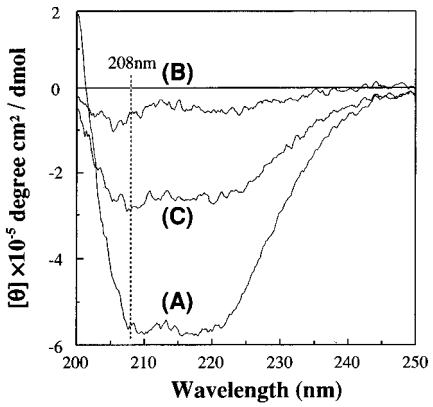

Figure 2. Circular dichroic spectra of untreated glucoamylase, (A) and glucoamylase treated with SC CO_2 (B) with and (C) without the micropore filter. Treatment conditions were set at 25 MPa and 35 °C for 30 min, and the pore size of the filter was 10 μm.

Comparison of SC CO_2 Inactivation with Heat Inactivation. The CD spectra of glucoamylase after SCT and heat treatment are shown in Figure 3. SCT was carried out at 25 MPa and 35 °C for 30 min and the heat treatment was carried out at 80 °C for 30 min. The residual activities of SC CO_2-treated and heat-treated glucoamylase were 17.5 and 0%, respectively.

Though the glucoamylase was thoroughly inactivated by the heat treatment, significant decay of the ellipticity

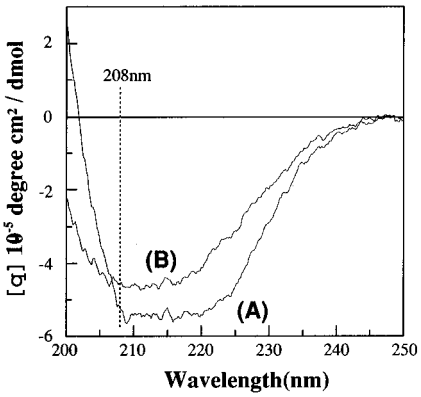

Figure 3. Circular dichroic spectra of (A) untreated glucoamylase and (B) glucoamylase treated with heat. The heat treatment was carried out at 80 °C for 30 min and atmospheric pressure.

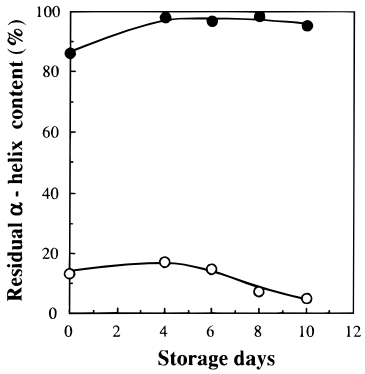

Figure 4. Changes in the α-helix contents of glucoamylases treated with SC CO_2 and heat during storage at 20 °C. Glucoamylase was treated with SC CO_2 at 35 °C and 25 MPa for 30 min (○) and treated with heat at 80 °C for 30 min and atmospheric pressure (●).

was not observed; that is, the α-helix structure remained at 85.7%. This result showed that heat inactivation would be accompanied by a slight change of the α-helix structure or the reversible production of the structure would occur almost instantly after the heat treatment.

To elucidate the reversible production of the α-helix structure, SC CO_2- and heat-treated glucoamylase samples were kept at 20 °C. Changes in the residual α-helix content are shown in Figure 4 (the conditions of SCT and heat treatment were the same as those in Figure 3). The residual α-helix content of glucoamylase treated with SC CO_2 decreased from 12.4 to 5.1% during 10 days and the residual activity also decreased from 17.5 to 0%. These results suggest that SCT would cause irreversible inactivation and a further conformational change that proceeded slowly even after SCT. In contrast, the residual α-helix content of heat-treated glucoamylase increased from 85.7 to 97.8% during the initial 4 days, and then no additional change was observed. Although the change in the secondary structure by heat treatment was almost reversible, the residual activity of heat-treated glucoamylase did not revert. It was concluded that the inactivation mechanism by SCT would be different from that of heat treatment.

Relationship between α-Helix Content and Enzyme Activity. To make clear the relationship between the enzyme activity and the conformational change, plots of the residual activities against the residual α-helix contents for glucoamylase and acid protease are shown in Figures 5 and 6, respectively. The

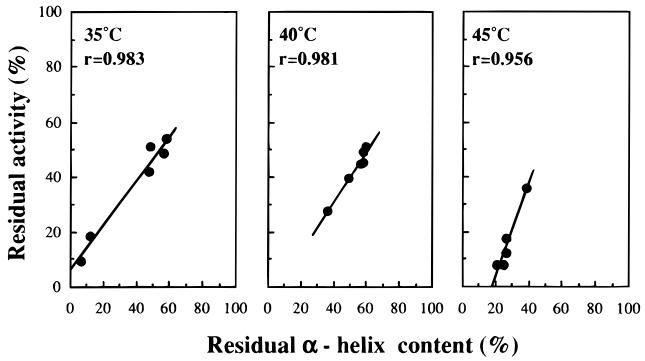

Figure 5. Relationship between residual enzyme activity and residual α-helix contents of glucoamylase. SCT was carried out at 8−30 MPa and 35−45 °C for 30 min.

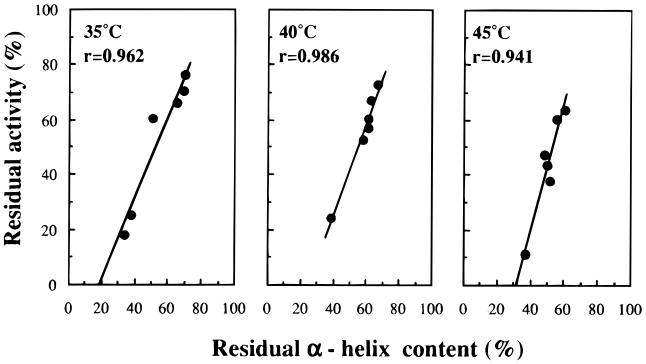

Figure 6. Relationship between residual enzyme activity and residual α-helix contents of acid protease. SCT was carried out at 8−30 MPa and 35−45 °C for 30 min.

SCT for each enzymes was carried out at 8−30 MPa and 35−45 °C for 30 min. The residual activities at these conditions have been shown in our previous paper (Ishikawa et al., 1995a). As a result, a close relationship was observed under each treatment temperature. The correlation coefficients for glucoamylase and acid protease were higher than 0.96 and 0.94, respectively. These results show that the decomposition of the α-helix structure would result in the loss of activity. Furthermore, the slope of the lines for both enzymes increased with increasing SCT temperature, suggesting that the enzyme were inactivated by the combined action of SCT and heating.

Chen et al. (1992) investigated the inactivation of polyphenol oxidases (PPOs) from lobster, brown shrimp, and potato by high-pressure (not supercritical-state) carbon dioxide. They also investigated the change of secondary structures of PPOs by CD spectra analysis, but the decay of negative ellipticity of CD spectra was smaller than that observed in our experiment. In addition, the changes of secondary structure varied in PPOs from the different origins and the authors did not relate the change of α-helix structures to the loss of activity.

The relationship between the structure and enzyme catalytic function has been studied on *Rhizopus* glucoamylase (Tanaka et al., 1986). This glucoamylase contains 11 α-helix structures, which exist in the C-terminal portion (135−604). This portion is more hydrophobic than the N-terminal portion and contributes to starch degradation. The sorption of SC CO_2 into the C-terminal portion with high hydrophobicity would cause decomposition of the α-helix structure and result in the loss of the enzyme catalytic function.

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Received for review March 29, 1996. Revised manuscript received June 24, 1996. Accepted July 3, 1996.[⊗]

JF9602075

[⊗] Abstract published in *Advance ACS Abstracts,* August 15, 1996.