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# Inactivation and structural change of horseradish peroxidase treated with supercritical carbon dioxide

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**Abstract**

The influence of supercritical carbon dioxide ($SCCO_2$) at 55 °C on inactivation of horseradish peroxidase (HRP) in buffer solution, pH 5.6, was studied while its structural change was analyzed by far UV-circular dichroism (CD) and tryptophan fluorescence spectroscopy. $SCCO_2$ treatment had significant effects on the residual activity of HRP, the least residual activity was only 12% at 30 MPa. HRP's secondary and tertiary structures were changed. The α-helix relative content in the secondary structure decreased and the intrinsic relative fluorescence intensity (RFI) increased as the pressure of $SCCO_2$ treatment was elevated. The HRP's inactivation closely corresponded to the loss of α-helix relative content and the increase of RFI. After a 7-day storage at 4 °C, the restoration of residual activity and the reversion of the α-helix relative content were observed while RFI resumed with exception of the 30 MPa treatment.


*Keywords:* Horseradish peroxidase; Inactivation; Supercritical carbon dioxide; Far UV-circular dichroism; Tryptophan fluorescence spectroscopy

## 1. Introduction

Non-thermal methods for the preservation of foods have been under intense investigation to evaluate their potential as an alternative or complementary process to traditional thermal methods (Mertens & Knorr, 1992). Non-thermal processes are expected to induce only minimum quality degradation of food and to keep flavour, colour, taste and nutrients of foods better.

The application of $SCCO_2$, devoted to inactivation of microorganisms, is under intense investigation. It was shown that $SCCO_2$ has significant lethal effect on microorganisms in liquid foods (Arreola et al., 1991; Ballestra & Cuq, 1998; Ballestra, Silva, & Cuq, 1996; Corwin & Shellhammer, 2002; Erkmen, 2000; Erkmen, 2001; Erkmen & Karaman, 2001; Hong & Pyun, 1999; Hong & Pyun, 2001; Kamihira, Taniguchi, & Kobayashi, 1987; Park, Lee, & Park, 2002; Shimoda et al., 1998, 2001). Furthermore, inactivations of enzymes subjected to $SCCO_2$ treatment is important. There have been reports about the inactivation of enzymes by $SCCO_2$. The inactivation of α-amylase, glucose oxidase, lipase and catalase activity by $SCCO_2$ treatment were studied and all treated enzymes retained over 90% activity (Taniguchi, Kamihara, & Kobayashi, 1987). Balaban et al. (1991) reported that pectin-esterase (PE) could be inactivated with $SCCO_2$. The total inactivation of lipoxygenase (LOX) and of peroxidase (POD) was achieved through $SCCO_2$ treatment of unbuffered solution (Tedjo, Eshtiaghi, & Knorr, 2000). Fadíloglu and Erkmen (2002) observed about 84% of initial activity of lipase was lost when exposed to $CO_2$ under atmospheric pressure treatment. The activity of polyphenol oxidases (PPOs) declined following high-pressure $CO_2$ treatment (Chen, Balaban, Wei, Marshall, & Hsu, 1992). Ishikawa, Shimoda, Yonekura, and Osajima (1996) reported that several enzymes, such as lipase, alkaline protease, acid protease,

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and glucoamylase, were inactivated and their α-helix structures were decomposed after $SCCO_2$ treatment.

Most researches have focussed on the inactivation of enzymes and little information is available on the inactivation mechanism and structural change of enzymes, due to the lack of enzyme structural data. Therefore, the $SCCO_2$ inactivation mechanism of enzymes, is not completely elucidated or understood.

Peroxidases (POD, E.G. 1.11.1.7) are widespread in plants, microbes, and animal tissues, and the peroxidase superfamily is classified into three groups on the basis of amino acid homology and metal-binding capabilities. Class I comprises intracellular peroxidases, including cytochrome *c* peroxidase, ascorbate peroxidase and the *S* gene-duplicated bacterial catalase-peroxidase. Class II contains the secretory fungal enzymes, such as manganese peroxidase and lignin peroxidase. Class III consists of the secretory plant peroxidase (Zamorano et al., 2004). Horseradish peroxidase (HRP) is an important heme-containing enzyme that has been studied for more than a century, belonging to class III of the plant peroxidase superfamily (Veitch, 2004), and its structure and biochemical properties have been characterized very well (Chattopadhyay & Mazumdar, 2000; Veitch, 2004). The only tryptophan residue present in class III/peroxidases was found in the extended structure connecting helices D and D′ and is likely a conserved residue (Carvalho et al., 2003). Their common features include Fe (III) protoporphyrin IX as the prosthetic group, a catalytic mechanism, conserving catalytic residues, a single tryptophan (Trp 117), four disulfide bonds, two $Ca^{2+}$ binding sites located distal and proximal to heme and eight glucans (Henriksen et al., 2001). The tryptophan fluorescence is quenched due to intramolecular tryptophan–heme energy; transfer in native HRP, which is weakened as the distance between the two groups increases. The tryptophan fluorescence intensity in HRP increases, probably due to the increase of the distance from heme group during denaturation and the quenching effect is less pronounced during denaturation of HRP as the distance between the tryptophan residue and heme group increases (Carvalho et al., 2003).

The far UV-CD and intrinsic tryptophan fluorescence spectrum carries information about the tertiary and secondary structure of the protein, changes in far UV-CD correspond to changes in the overall secondary structure of the protein, while changes in intrinsic tryptophan fluorescence emission correspond to changes in the tertiary structure of the protein (Zamorano et al., 2004). The changes in secondary and tertiary structure of HRP induced by a pulsed electric field were studied by these two methods in our laboratory (Zhong, Hu, Zhao, Chen, & Liao, 2005).

The purpose of this study is to investigate the influence of $SCCO_2$ treatment on the the residual activity and the structural change of HRP.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Enzyme

Horseradish peroxidase (abbreviated as HRP, RZ > 3, 300 U/mg) was purchased from Shanghai Xueman Biotechnology Co. (Shanghai, China). The enzyme was dissolved in (pH 5.6) acetate buffer to a concentration of 2.5 μg/ml and subjected to $SCCO_2$ treatment and thermal inactivation.

#### 2.1.2. Carbon dioxide

The purity of $CO_2$ is 99.9%, which was purchased from Beijing Analytical Apparatus Co. (Beijing, China) and was purified with an activated carbon column (1 m in length and 2 cm in diameter).

#### 2.1.3. Reagents

Guaiacol and hydrogen were purchased from Beijing Chemicals Co. (Beijing, China). All other chemicals in the investigation were of analytical grade.

### 2.2. $SCCO_2$ treatment system

$SCCO_2$ treatment was performed with a system (designed by Tsinghua University, Beijing, China). A schematic diagram of the system used in this study is shown in Fig. 1. This system consisted of a 200 ml stainless steel pressure vessel, temperature controllers, pressure gauge and two plunger-type pumps. The system pressure was controlled by a back-pressure regulator and indicated by pointer manometers with an accuracy of 0.4%. The copper-constantan needle-type thermocouple was placed inside the vessel to monitor the temperature. An electrical heating jacket was placed around the vessel. Another thermocouple connected to a temperature controller was placed between the outer surface of the vessel and the inner surface of the heating jacket to control and maintain a constant temperature. The temperature fluctuation was ±1 °C during the treatment. The minimum pressure was chosen to be slightly above the critical pressure for $CO_2$ (8 MPa) and the maximum pressure was slightly less than the operating limit of the equipment (35 MPa). The applied temperature in the study was 55 °C and the treatment time was 60 min.

### 2.3. HRP activity measurement

HRP activity assays were performed using a substrate solution that contained 0.057 ml of 30% hydrogen peroxide and 0.056 ml of liquid guaiacol in 100 ml of 0.1 M (pH 5.6) acetate buffer. HRP activity was determined by adding 0.1 ml of sample, appropriately diluted, to 3.0 ml of substrate solution equilibrated at 30 °C for 5 min. The rate of increase in absorbance at

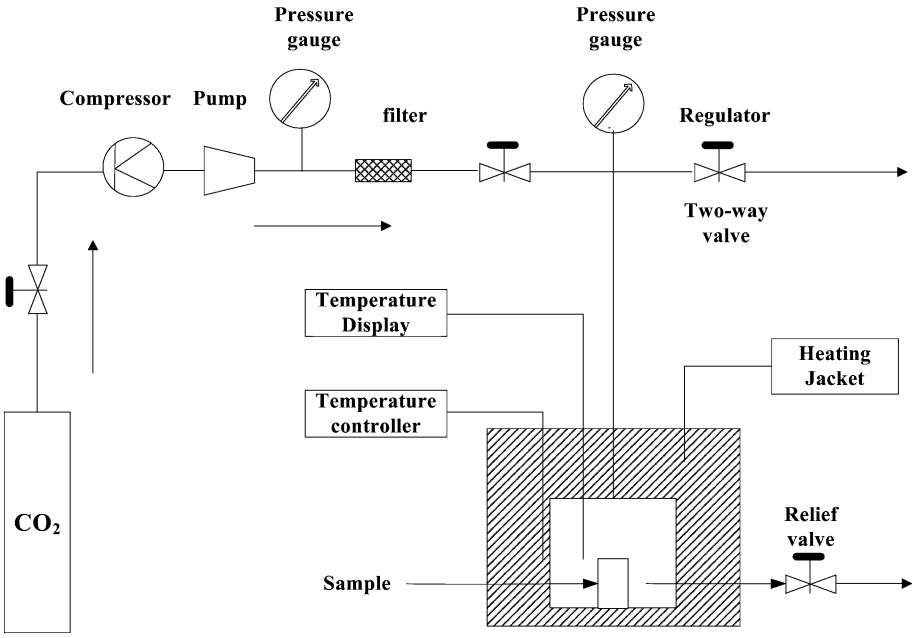


Fig. 1. Schematic diagram of supercritical carbon dioxide processing equipment.

470 nm, due to the formation of brown guaiacol oxidation products, was measured spectrophotometrically using a UV-762 spectrophotometer (Lingguang, Shanghai, China) at ambient temperature (25 ± 1 °C). The specific activity was calculated from the slope of a linear segment of at least 30 s duration, expressed as $A_{470\ nm}$/min/0.1 ml of undiluted sample. The residual activity of HRP were obtained with the following formula.

$$\text{Residual activity} = \frac{\text{specific activity of HRP treated with SCCO}_2 \times 100\%}{\text{specific activity of HRP before SCCO}_2}.$$

### 2.4. Thermal inactivation of HRP

Five milliliters HRP sample were placed in a 18 × 180 mm glass tube (Beijing Bomex company, Beijing, China) and heated in a water bath at 100 °C for 30 min for a complete inactivation. The heating time was counted after the temperature of HRP sample reached the desired temperature. After the treatment, the tube was removed and cooled in an ice bath. Following equilibration to ambient temperature, the residual activity were determined.

### 2.5. $SCCO_2$ inactivation of HRP

For each experiment, 1.5 ml of HRP sample were placed in a 5 ml of Micro Test Tube (Beijing Bomex company, Beijing, China) without the cap and then placed in the $SCCO_2$ vessel which had been preheated to the experimental temperature, and then was pressurized by $SCCO_2$ treatment system. The $CO_2$ was fed for about 5–10 min until the pressure reached the experimental level, and then the feeding was stopped. The sample was held at the constant pressure and temperature during the treatment. At the end of the $SCCO_2$ treatment, the vessel was slowly depressurized by releasing the pressure valve over a period of 15 min. After treatment, the sample was removed and immediately cooled in an ice bath. Following equilibration to ambient temperature, the residual activity was determined.

### 2.6. Restoration of HRP activity

To examine the restoration ability of HRP following $SCCO_2$ treatment, some HRP solution samples treated by $SCCO_2$ and HRP solution control samples without $SCCO_2$ treatment were kept at 4 °C for 3 weeks, followed by measuring the activity of HRP. The assays of HRP activity were performed weekly as previously described. The percentage of residual activity was determined as $AR_t/AR'_t \times 100$, where $AR_t$ represented the activity of $SCCO_2$-treated HRP kept at time $t$ and $AR'_t$ the activity of $SCCO_2$-untreated control HRP kept at time $t$.

### 2.7. Circular dichroism (CD) analysis of HRP

CD spectra were recorded with a JASCO J-720 CD spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan), using a quartz cuvette of 1 mm optical path length at ambient temperature (25 ± 1 °C). CD spectra were scanned in the far UV range (250–200 nm) with four replicates at 50 nm/min, bandwidth = 1 nm. The CD data were expressed in terms of mean residue ellipticity ($\theta$) in deg cm$^2$ dmol$^{-1}$. The HRP concentrations

for CD analysis were 4.55 μM. The CD spectra of all the samples are scanned immediately after treatment and after a 7-day storage at 4 °C. All CD spectra measured were baseline corrected by the buffer, the secondary structure elements of HRP were computed from the data using the computer K2D programme (Andrade, Chacón, Merelo, & Morán, 1993).

$$\text{Relative } \alpha\text{-helix content} = \frac{\text{content of } \alpha\text{-helix after treatments} \times 100\%}{\text{content of } \alpha\text{-helix before treatments}}.$$

### 2.8. Tryptophan fluorescence spectroscopy analysis of HRP

Tryptophan fluorescence spectra were, measured with a HITACHI F-4500/spectrofluorometer (HITACHI, Japan), using a quartz cuvette of 1 cm optical path length at ambient temperature (25 ± 1 °C). All the samples were observed immediately after treatment and after storage for 7 days at 4 °C. The HRP concentration was 3.41 μM The emission spectra ($\lambda_{em}$ from 300 to 450 nm) were obtained at the maximum excitation wavelength $\lambda_{ex} = 293$ nm and represented the mean of three scans.

$$\text{Relative fluorescence intensity} = \frac{\text{fluorescence intensity after } SCCO_2}{\text{fluorescence intensity before } SCCO_2}.$$

### 2.9. Statistical analysis

Analyses of variance (ANOVA) were carried out by using the software Microcal Origin 6.0 (Microcal Software, Inc., Northampton, USA). The ANOVA test was performed for all experimental runs to determine significance at 95% confidence interval. All experiments were performed in triplicate.

## 3. Results and discussion

### 3.1. Inactivation and restoration of HRP activity subjected to $SCCO_2$ treatment

The inactivation and restoration of HRP residual activity subjected to $SCCO_2$ treatment is illustrated in Fig. 2. The temperature of HRP solution was maintained, invariably, at 55 °C. After $SCCO_2$ treatment, the loss of the residual activity of HRP was significant ($p = 0.05$) as compared to control sample HRP, the least residual activity of HRP was 12%, reached at 30 MPa, as the applied pressure of $SCCO_2$ treatment was increased, the residual activity of HRP was reduced, indicating that the higher the pressure of $SCCO_2$ treatment, the greater was the loss of HRP residual activity, the residual activity of HRP was closely related to the pressure of $SCCO_2$ treatment. Analysis of variance (ANOVA) results indicated that $SCCO_2$ treatment had significant effects on the relative activity of HRP.

Meanwhile, the restoration of HRP residual activity was also observed in our study. After storage for 7 and 21 days at 4 °C, HRP solution exhibited a higher residual activity than that measured immediately after $SCCO_2$ treatment, implying that the restoration of HRP residual activity occurred. Analysis of variance (ANOVA) shows that the restorative ability of HRP

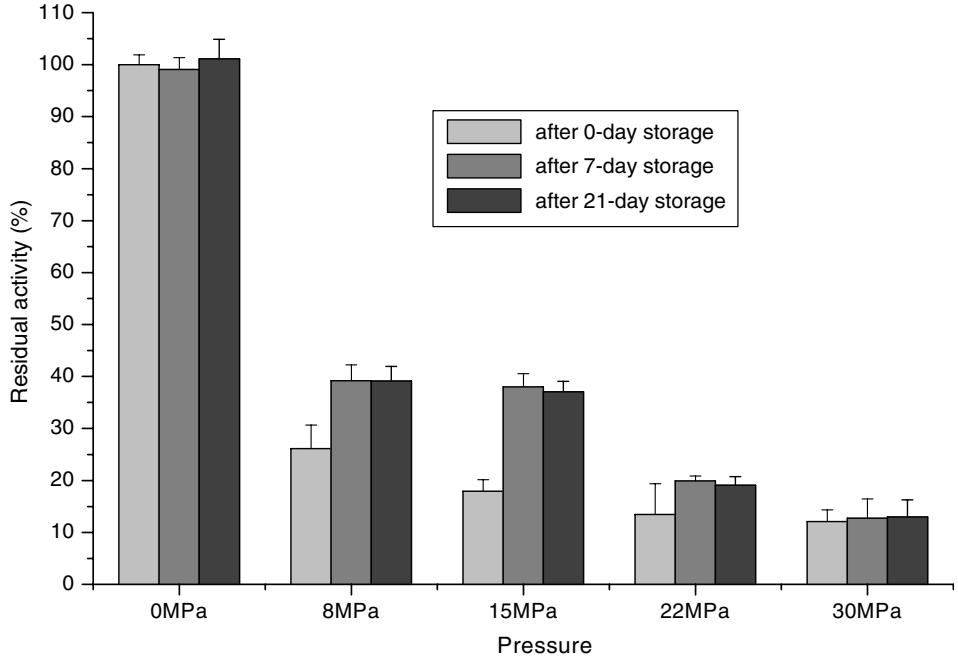


Fig. 2. Residual activity of HRP as a function of $SCCO_2$ treatment pressure at 55 °C.

subjected to $SCCO_2$ treatment at 8, 15 and 22 MPa was significant and, at 30 MPa, it was not obvious after a 7-day storage at 4 °C, and then a small drop of HRP residual activity after a 21-day storage at 4 °C this change was not significant ($p = 0.05$).

Earlier reports were not completely consistent. Chen et al. (1992) reported that potato PPO treated by high pressure $CO_2$ regained 28% of the original activity during the first 2 weeks of frozen storage. Residual activity of pectinesterase in orange juice also exhibited a slow increase of activity after storage for 15 days at 4 °C, regardless of the pressure and temperature during the treatment (Balaban et al., 1991). Machado and Saraiva (2002) found that, after a steep increase of the HRF activity, an equilibrium stage was attained where the values of the enzyme activity were not significantly increased with increasing time of storage after thermal inactivation. But Ishikawa et al. (1996) showed that the residual activity of glucoamylase treated with $SCCO_2$ decreased during 10 days at 20 °C.

The residual activity of heat-treated HRP was completely lost and did not restore after storage for 7 and 21 days at 4 °C (not shown in Fig. 2).

### 3.2. CD spectra analysis of HRP by $SCCO_2$

The far UV-CD spectra of all the samples were measured immediately after treatment and after a 7-day

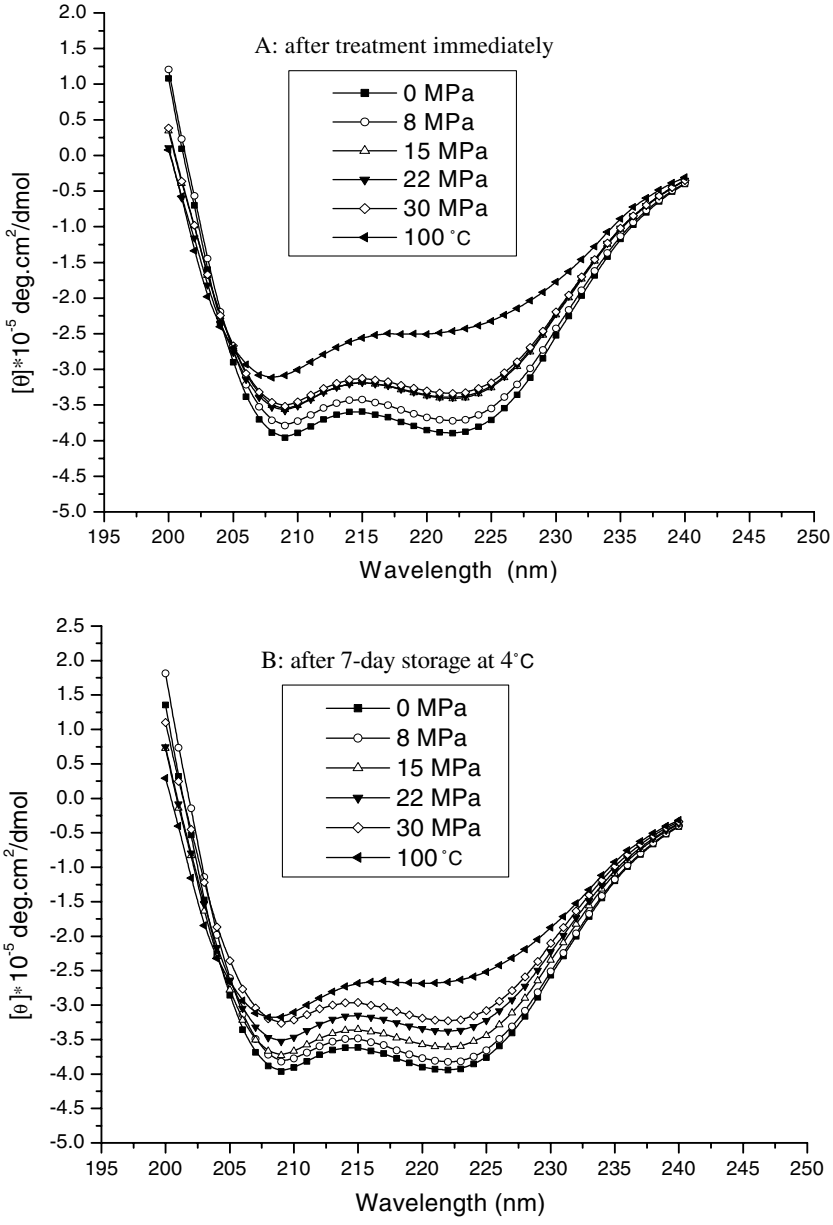


Fig. 3. Far UV-CD spectra of $SCCO_2$-treated and heat-treated HRP. HRP concentration was 4.55 μM. (A) All samples were measured immediately after treatment; (B) all samples were measured after 7-day storage at 4 °C.

storage at 4 °C. Fig. 3A presents the CD spectra of HRP in the region of peptide bond absorption (200–250 nm) as a function of $SCCO_2$ pressures at 55 °C. It is commonly known that two negative peaks, at 208 and 222 nm, are characteristic of a protein with a large amount of α-helix secondary structure (Chang, Wu, & Yang, 1978; Myer, 1968), and their intensity reflects the amount of helical structure in protein (Venyaminov & Yang, 1996). As shown in Fig. 3A, HRP had double negative peaks in the far UV-CD spectra at around 208 and 222 nm. This observation was similar to earlier reports (Amishka Kamal & Behere, 2001; Tang et al., 2002; Carvalho et al., 2003). The fraction of secondary structure elements was calculated by using a K2D programme, with 57% α-helix, 8% β-sheet and others 34%. Veitch (2004) reported that the structure of the enzyme was largely α-helical although there was also a small region (β-sheet).

After treatment with $SCCO_2$ and heat, the intensities of two negative peaks in the CD spectra of HRP decreased, indicating a loss of the α-helix structure fraction of HRP; this result showed that these treatments caused conformational changes in the secondary structures. As HRP was completely inactivated at 100 °C for 30 min, an obvious decrease of the ellipticity was observed. The ellipticity of HRP treated by $SCCO_2$ gradually decreased as the pressure level of $SCCO_2$ treatment increased; higher $SCCO_2$ pressure resulted in greater decrease in the two negative peaks, indicating α-helix conformation reduction; this corresponded nicely to the loss of the above HRP residual activity in Fig. 2. Fig. 4 presents the α-helix relative content of HRP, measured immediately after treatment and after a 7-day storage at 4 °C. After the $SCCO_2$ treatments at 8, 15, 22 and 30 MPa with 55 °C for 60 min, the residual α-helix relative content of those samples decreased to 91.23%, 85.96%, 84.21% and 82.46%, respectively. This difference in the responsiveness of the α-helix secondary structure to $SCCO_2$ treatment could account for the above finding that higher $SCCO_2$ a pressure resulted in greater reduction of HRP residual activity. These results suggest that the reduction of HRP activity could be related to the destruction of HRP secondary structure caused by $SCCO_2$. Chen et al. (1992) showed that high pressure $CO_2$ treatment caused conformational changes of PPOs from lobster, shrimp and potato in the secondary structure suggested by CD analysis. Therefore, the inactivation of HRP was related to the conformational change of α-helix induced by $SCCO_2$ treatment and thermal treatment. To elucidate the relationship between the residual activity of enzymes and α-helix relative content in enzymes, that the residual activity. against residual α-helix relative content could be plotted. Ishikawa et al. (1996) reported that the residual activity of glucoamylase and acid protease subjected to $SCCO_2$ treatment had a good linear relationship with the residual α-helix relative content and their $R$ values were greater than 0.96 and 0.94. But in our study, the relationship could not be quantified because the HRP concentrations were different in the residual activity measurement and CD analysis.

As shown in Fig. 3B, the CD spectra of HRP subjected to $SCCO_2$ treatment after a 7-day storage were essentially similar, but the ellipticity at the maxima of 208 and 222 nm decreased, corresponding to that measured immediately after $SCCO_2$ treatment with exception of the control HRP solution, implying that the α-helix content increased after a 7-day storage. After

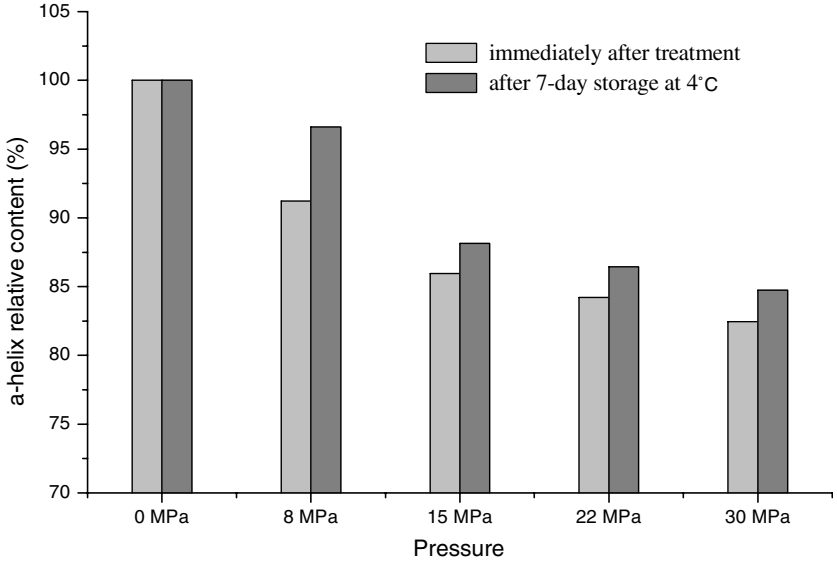


Fig. 4. α-Helix Relative content of HRP immediately after treatments and after storage for 7 days at 4 °C. HRP concentration was 4.55 μM.

a 7-day storage at 4 °C, the α-helix structure relative content of those samples returned 96.61%, 88.14%, 86.44% and 84.75% in Fig. 4, respectively. The reversion of the α-helix structure of HRP closely corresponded to the restoration of HRP residual activity observed during the 7-day storage at 4 °C in Fig. 2. It was concluded that the regain of α-helix relative content was beneficial to the restoration of the residual activity of HRP. This result was not consistent with the previous observation that the residual α-helix content of glucoamylase treated with $SCCO_2$ exhibited a significant decrease with a 10-day storage at 20 °C (Ishikawa et al., 1996).

The residual α-helix content of HRP treated at 100 °C for 30 min decreased to 70.18% as compared to untreated HRP, and increased to 74.58% after a 7-day storage (not shown in Fig. 4), but no restoration of the residual activity of HRP was measured. A similar observation was reported earlier, Ishikawa et al. (1996) found that the residual α-helix structure of heat-treated glucoamylase increased during the initial 4 days with no reactivation of HRP activity. The inactivation mechanism by $SCCO_2$ would be different from that of heat treatment (Ishikawa et al., 1996).

### 3.3. Tryptophan fluorescence spectrum analysis of HRP by $SCCO_2$

Fluorescence spectroscopy has been shown to be a very useful technique for studying structure and dynamics in protein. Environmental changes resulting from conformational changes in the tertiary structure of proteins can be measured by intrinsic fluorescence spectroscopy (Zamorano et al., 2004).The intrinsic fluorescence emission from tryptophan residue in proteins was an in-built probe and was sensitive to the microenvironment surrounding the fluorophore residue (Amishka Kamal &

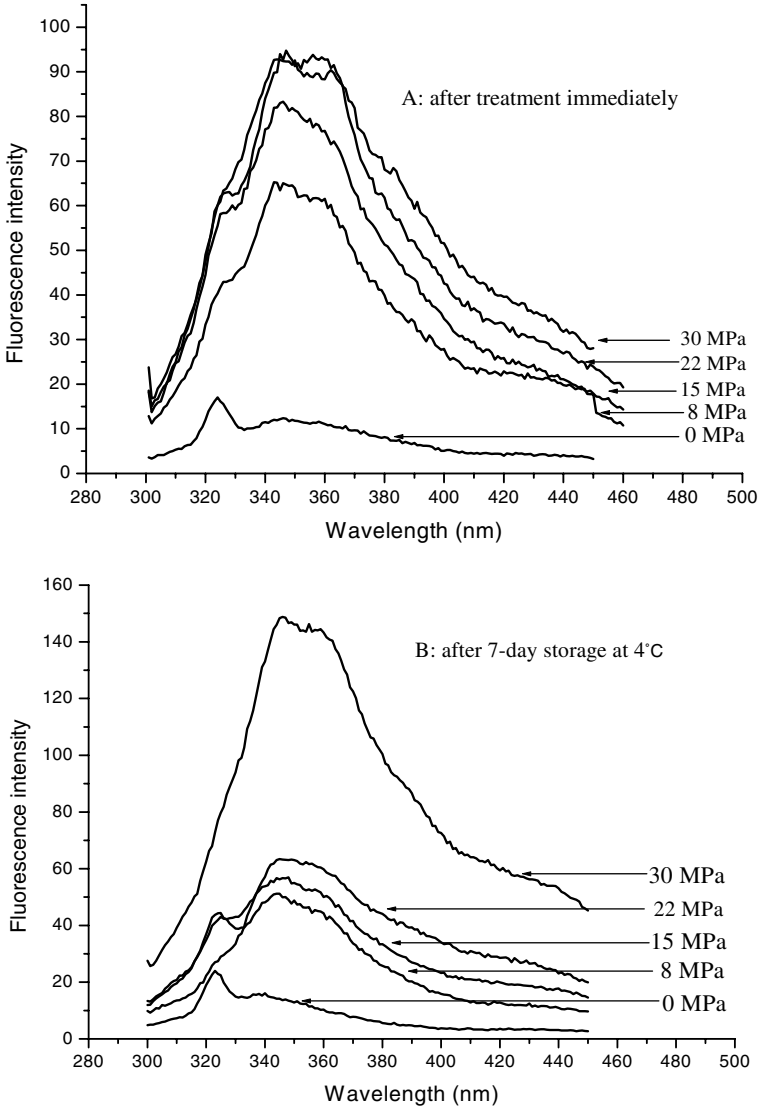


Fig. 5. Fluorescence emission spectra of $SCCO_2$-treated and heat-treated HRP. HRP concentration was 3.41 μM. (A) All samples were measured immediately after treatment; (B) all samples were measured after 7-day storage at 4 °C.

Behere, 2001). Therefore, the change in the tertiary structure of HRP protein could be determined by intrinsic emission spectroscopy and fluorescence intensity.

Fig. 5A and B depict the fluorescence emission spectra of HRP at the excitation wavelength of 293 nm (determined by the present study). All samples were measured immediately after treatment and after a 7-day storage at 4 °C. The fluorescence emission intensity of untreated HRP was very weak, because the tryptophan fluorescence was quenched due to intramolecular tryptophan–heme energy transfer in native HRP (Carvalho et al., 2003; Zamorano et al., 2004). The fluorescence emission spectra of HRP subjected to $SCCO_2$ treatment showed that a great change of the emission maxima and the maximum fluorescence intensity had occurred, indicating change in the tertiary structure of HRP. The emission maximum wavelength, $\lambda_m$, of untreated HRP was observed at 324 nm, while the emission maximum wavelength, $\lambda_m$, of $SCCO_2$-treated HRP was increased to around 345 nm; this wide span of red-shift was approximately 21 nm, indicating that the tryptophan surroundings changed to a more polar environment upon $SCCO_2$ treatment. This observation was similar to the finding of Carvalho et al. (2003) in a study of thermal denaturation of HRP. Peak fluorescence intensity was enhanced correspondingly as the pressure level of $SCCO_2$ treatments was elevated, higher pressure resulting in higher fluorescence intensity. Fig. 6 presents the change of the HRP relative fluorescence intensity (RFI) at the emission maximum $\lambda_m$ 345 nm, which was observed immediately after treatment and for 7 days of storage at 4 °C, respectively. After the $SCCO_2$ treatment at 8, 15, 22 and 30 MPa at 55 °C, the RFI increased sharply and reached 524.722%, 672.028%, 748.113%, 745.177% in the fluorescence spectra. As the pressure level of the $SCCO_2$ treatment was elevated, the tryptophan fluorescence intensity of HRP increased but the difference was slight, accompanied by a decrease of residual activity HRP. The tryptophan fluorescence intensity in HRP increased, probably due to the increase of the distance from the heme group during denaturation (Carvalho et al., 2003). It was concluded that $SCCO_2$ treatment was beneficial to increase the distance between the tryptophan and the heme group.

After a 7-day storage at 4 °C, the fluorescence emission spectra of HRP did not alter, as essentially illustrated in Fig. 5B, but the maximum fluorescence intensity of HRP subjected to 8, 15 and 22 MPa treatments reversed partly or declined to 366.64%, 415.8%, 464.153% (in Fig. 6) significantly while it still increased greatly 1091.433% at 30 MPa treatment (in Fig. 6) and the maximum fluorescence intensity of the control HRP remained almost constant. Although the alteration, depending on the pressure levels, was a little contradictory, but it could explain that the restorative ability of HRP subjected to $SCCO_2$ treatment at 8, 15 and 22 MPa was significant and it was not obvious at 30 MPa after a 7-day storage at 4 °C (in Fig. 2).

Interestingly, we also found that the emission maximum existed at 584 nm as shown in Fig. 7A, as the pressure of $SCCO_2$ treatment increased, the intensity of the emission spectra was increased (Fig. 7B). This present study could not explain how this related to structural change.

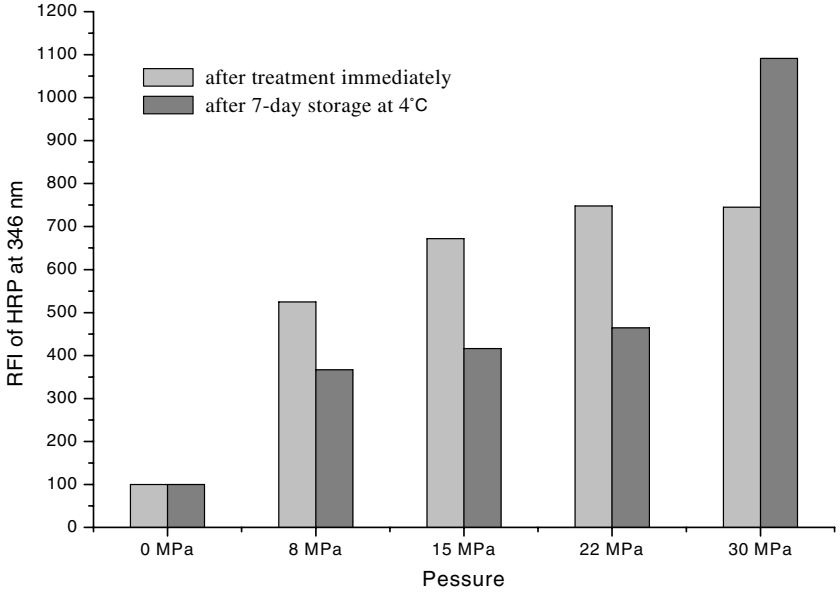


Fig. 6. Intrinsic relative fluorescence intensity (RFI) of HRP at 345 nm immediately after treatments and after storage for 7 days at 4 °C. HRP concentration was 3.41 μM.

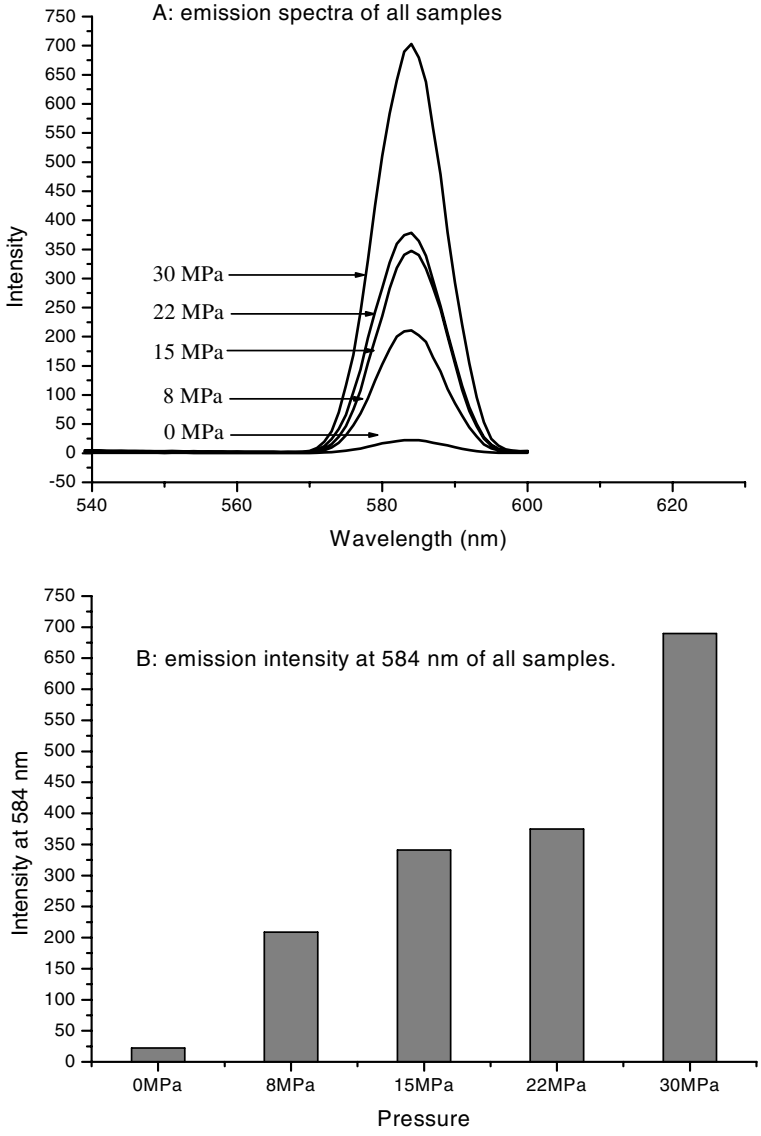


Fig. 7. Emission spectra of HRP and intrinsic fluorescence intensity at 584 nm of HRP measured immediately after $SCCO_2$-treatment and control HRP. HRP concentration was 3.41 μM. (A) Emission spectra of all samples; (B) emission intensity at 584 nm of all samples.

## 4. Conclusion

In this study, $SCCO_2$ treatment had significant effects on the residual activity of HRP, and the higher the pressure of $SCCO_2$ treatment, the greater was the loss of HRP residual activity, the least residual activity of HRP being only 12% at 30 MPa. The residual activity of HRP was closely related to the pressure of $SCCO_2$ treatment. The secondary structure and the tertiary structure of HRP after $SCCO_2$ treatment changed as suggested by CD analysis and fluorescence spectroscopy analysis, respectively. The α-helix relative content in the secondary structure of HRP decreased and the intrinsic relative fluorescence intensity (RFI) increased as the pressure of $SCCO_2$ treatment was elevated. The inactivation of $SCCO_2$ treated HRP closely corresponded to the loss of, α-helix relative content and the increase of RFI. After a 7-day storage at 4 °C, the restoration of HRP residual activity and the reversion of the α-helix relative content were also observed, while the intrinsic relative fluorescence intensity resumed, with the exception of 30 MPa treatment.

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## References


Amishka Kamal, J. K., & Behere, D. V. (2001). Steady-state and picosecond time-resolved fluorescence studies on native and apo seed coat soybean peroxidase. *Biochemical and Biophysical Research Communications, 289*, 427–433.

Andrade, M. A., Chacón, P., Merelo, J. J., & Morán, F. (1993). Evaluation of secondary structure of proteins from UV circular dichroism using an unsupervised learning neural network. *Protein Engineering, 6*, 383–390.

Arreola, A. G., Balaban, M. O., Wei, C. I., Peplow, A. J., Marshall, M., & Cornell, J. (1991). Effect of supercritical carbon dioxide on microbial populations in single strength orange juice. *Journal of Food Quality, 14*, 275–284.

Balaban, M. O., Arreola, A. G., Marshall, M., Peplow, A., Wei, C. I., & Cornell, J. (1991). Inactivation of pectinesterase in orange juice by supercritical carbon dioxide. *Journal of Food science, 56*, 743–746.

Ballestra, P., & Cuq, J. L. (1998). Influence of pressurized carbon dioxide on the thermal inactivation of bacterial and fungal spores. *Lebensmittel-Wissenschaft und-Technologie, 31*, 84–88.

Ballestra, P., Silva, A. A. D., & Cuq, J. L. (1996). Inactivation of *Escherichia coli* by carbon dioxide under pressure. *Journal of Food Science, 61*, 829–831.

Carvalho, A. S. L., Melo, E. P. E., Ferreira, B. S., Neves-Petersen, M. T., Petersen, S. B., & Aires-Barros, M. J. R. (2003). Heme and pH-dependent stability of an anionic horseradish peroxidase. *Archives of Biochemistry and Biophysics, 415*, 257–267.

Chang, C. T., Wu, C. S. C., & Yang, J. T. (1978). Circular dichroic analysis of protein conformation: inclusion of the b-tums. *Analytical Biochemistry, 91*, 13–31.

Chattopadhyay, K., & Mazumdar, S. (2000). Structural and conformational stability of horseradish peroxidase: effect of temperature and pH. *Biochemistry, 39*, 263–270.

Chen, J. S., Balaban, M. O., Wei, C. L., Marshall, M. R., & Hsu, W. Y. (1992). Interaction of polyphenol oxidase by high pressure carbon dioxide. *Journal of Agricultural and Food Chemistry, 40*, 2345–2349.

Corwin, H., & Shellhammer, T. H. (2002). Combined carbon dioxide and high pressure inactivation of pectin methylesterase, polyphenol oxidase, *Lactobacillus plantarum* and *Escherichia coli*. *Journal of Food Science, 67*, 697–701.

Erkmen, O. (2000). Inactivation of *Salmonella typhimurium* by high pressure carbon dioxide. *International Journal of Food Microbiology, 17*, 225–232.

Erkmen, O. (2001). Effects of high-pressure carbon dioxide on *Escherichia coli* in nutrient broth and milk. *International Journal of Food Microbiology, 65*, 131–135.

Erkmen, O., & Karaman, H. (2001). Kinetic studies on the high pressure carbon dioxide inactivation of *Salmonella typhimurium*. *Journal of Food Engineering, 50*, 25–28.

Fadíloglu, S., & Erkmen, O. (2002). Inactivation of lipase by carbon dioxide under atmospheric pressure. *Journal of Food Engineering, 52*, 331–335.

Henriksen, A., Mirza, O., Indiani, C., Teilum, K., Smulevich, G., Welinder, K. G., & Gajhede, M. (2001). *Protein Science, 10*, 108–115.

Hong, S. I., & Pyun, Y. R. (1999). Inactivation kinetics of *Lactobacillus plantarum* by high pressure carbon dioxide. *Journal of Food Science, 64*, 728–733.

Hong, S. I., & Pyun, Y. R. (2001). Membrane damage and enzyme inactivation of *Lactobacillus plantarum* by high pressure $CO_2$ treatment. *International Journal of Food Microbiology, 63*, 19–28.

Ishikawa, H., Shimoda, M., Yonekura, A., & Osajima, Y. (1996). Inactivation of enzymes and decomposition of α-helix structure by supercritical carbon dioxide microbubble method. *Journal of Agricultural and Food Chemistry, 44*, 2646–2649.

Kamihira, M., Taniguchi, M., & Kobayashi, T. (1987). Sterilization of microorganisms with supercritical carbon-dioxide. *Agricultural and Biological Chemistry, 51*, 407–412.

Machado, M. F., & Saraiva, J. (2002). Inactivation and reactivation kinetics of horseradish peroxidase in phosphate buffer and buffer-dimethylformamide solution. *Journal of Molecular Catalysis B: Enzymatic, 19–20*, 451.

Mertens, B., & Knorr, D. (1992). Development of nonthermal processes for food preservation. *Food Technology, 46*, 124–133.

Myer, Y. P. (1968). Conformation of cytochromes II. Comparative study of circular dichroism spectra, optical rotatory dispersion, and absorption spectra of horse heart cytochrome. *Biology Chemistry, 243*, 2115–2122.

Park, S. I., Lee, J. I., & Park, J. (2002). Effects of a combined process of high pressure carbon dioxide and high hydrostatic pressure on the quality of Carrot juice. *Journal of Food Science, 67*, 1827–1834.

Shimoda, M., Cocunubo-Castellanos, J., Kago, H., Miyake, M., Osajima, Y., & Hayakawa, I. (2001). The influence of dissolved $CO_2$ concentration on the death kinetics of *Saccharomyces cerevisiae*. *Journal of Applied Microbiology, 91*, 306–311.

Shimoda, M., Yamamoto, Y., Cocunubo-Castellanos, J., Tonoike, H., Kawano, T., Ishikawa, H., et al. (1998). Antimicrobial effects of pressured carbon dioxide in a continuous flow system. *Journal of Food Science, 63*, 709–712.

Tang, J., Jiang, J., Song, Y., Peng, Z., Wu, Z., Dong, S., & Wang, E. (2002). Conformation change of horseradish peroxidase in lipid membrane. *Chemistry and Physics of Lipids, 120*, 119–129.

Taniguchi, M., Kamihara, M., & Kobayashi, T. (1987). Effect of treatment with supercritical carbon dioxide on enzymatic activity. *Agricultural Biology and Chemistry, 51*, 593–594.

Tedjo, W., Eshtiaghi, M. N., & Knorr, D. (2000). Impact of supercritical carbon dioxide and high pressure on lipoxygenase and peroxidase activity. *Journal of Food Science, 65*, 1284–1287.

Veitch, N. C. (2004). Horseradish peroxidase: a modern view of a classic enzyme. *Phytochemistry, 65*, 249–259.

Venyaminov, S. Y., & Yang, J. T. (1996). Determination of protein secondary conformation. In G. D. Fasman (Ed.), *Circular dichroism and the conformational analysis of biomolecules* (pp. 69–107). New York and London: Plenum Press.

Zamorano, L. S., Pina, D. G., Gavilanes, R., Roig, M. G., Sakharov, I. Y., Jadan, A. P., et al. (2004). Two-state irreversible thermal denaturation of anionic peanut (*Arachis hypogaea* L.) peroxidase. *Thermochimica Acta, 417*, 67–73.

Zhong, K., Hu, X. S., Zhao, G. H., Chen, E., & Liao, X. J. (2005). Inactivation and conformational change of horseradish peroxidase induced by pulsed electric field. *Food Chemistry, 92*, 473–479.