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Inactivation of polyphenol oxidases in cloudy apple juice exposed to supercritical carbon dioxide

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Abstract

The inactivation of polyphenol oxidase (PPO) in cloudy apple juice exposed to supercritical carbon dioxide ($SCCO_2$) treatment was investigated. Higher pressure, higher temperature, and longer treatment time caused more inactivation of PPO. The maximum reduction of PPO activity reached more than 60% at 30 MPa and 55 °C for 60 min. The experimental data followed first-order reaction kinetics; the kinetic rate constant k and the decimal reduction time D were closely related to the pressure and temperature of $SCCO_2$ treatment. Higher pressures or higher temperatures resulted in lower D values (higher k), the D value of PPO was minimized to 145 min treated by the combination of 30 MPa and 55 °C. Activation energy of 18.00 kJ /mol, was significantly reduced by $SCCO_2$ treatment at 30 MPa, as compared to activation energy of 72.0 kJ/mol for identical treatment at atmospheric pressure. Pressure and temperature sensitivity of kinetic parameters were studied. Z_P at 55 °C was 66.7 MPa and Z_T at 30 MPa was 108 °C.

Keywords: Polyphenol oxidase; Cloudy apple juice; Supercritical carbon dioxide; Inactivation; First-order reaction

1. Introduction

Consumer demands for safe and minimally processed foods with high quality attributes have encouraged the food industry and academic researchers for finding innovative food processing (Farr, 1990; Mertens, 1992; Riahi & Ramaswamy, 2004). 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 (Heinz, Toepfl, & Knorr, 2003; Mertens & Knorr, 1992; Stewart, Tompkin, & Cole, 2002).

As a novel non-thermal method for the pasteurization of microorganisms and the inactivation of enzymes, treated with supercritical carbon dioxide ($SCCO_2$) is attracting much interest in the food industry. It was shown that $SCCO_2$ had significant lethal effect on microorganisms in liquid foods (Ballestra & Cuq, 1998; Ballestra, Silva, & Cuq, 1996; Hong & Pyun, 2001; Shimoda et al., 1998; Corwin & Shellhammer, 2002; Erkmen & Karaman, 2001; Park, Lee, & Park, 2002; Shimoda et al., 2001). Meanwhile, recent reports have dealt with the influence of $SCCO_2$ on enzymes. Balaban et al. (1991) reported that pectin–esterase (PE) in orange juicie could be inactivated with $SCCO_2$ and its D value (decimal reduction time) was 10 min at 31 MPa and 60 °C. Tedjo, Eshtiaghi, and Knorr (2000) observed that the total inactivation of lipoxygenase (LOX) and that of peroxidase (POD) was achieved through $SCCO_2$ treatment in unbuffered solution. However, Taniguchi, Kamihara, and Kobayashi (1987) found that over 90% of activities of α-amylase, glucose oxidase, lipase and catalase were retained after $SCCO_2$ treatment. The inactivation of enzyme exposed to $SCCO_2$ treatment depends on the type and source of enzyme, nature of the medium in which the enzyme is dispersed, pressure, temperature and treatment time (Chen, Balaban, Wei,

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Marshall, & Hsu, 1992; Taniguchi et al., 1987; Tedjo et al., 2000).

The inactivation of enzymes exposed to $SCCO_2$ treatment can be explained by the fact that $SCCO_2$ causes conformational changes in the secondary and tertiary structure. Ishikawa, Shimoda, Yonekura, and Osajima (1996) reported that several enzymes, such as lipase, alkaline protease, acid protease, and glucoamylase were inactivated and their α-helix structures were decomposed after $SCCO_2$ treatment. Chen et al. (1992) found that the activity of polyphenol oxidase (PPO), purified from Florid spiny lobster, broen shrimp and potato, declined and the secondary structures of PPO were changed following high-pressure CO_2 treatment. Our previous experiment also confirmed the conformation change of horseradish peroxidase in acetic buffer solution after $SCCO_2$ treatment (Gui et al., 2006).

Cloudy apple juice has increased market value due to its sensory and nutritional qualities, and it is expected to have the yellowish colour which characterizes the fresh product (Lozano, Drudis-Biscari, & Ibarz-Ribas, 1994; Ozoglu & Bayindirli, 2002). However, enzymatic browning, which is caused by the action of ployphenol oxidase (PPO), is a major problem for the cloudy apple juice, since it cause deleterious changes in organoleptic properties of the juice (Ozoglu & Bayindirli, 2002). Polyphenol oxidase (PPO, E.C. 1.10.3.1) is a Cu-containing enzyme, which is also known as catechol oxidase, catecholase, diphenol oxidase, *o*-diphenolase, phenolase and tyrosinase (Martinez & Whitaker, 1995). Several methods, such as thermal treatment, the addition of antioxidants and high pressure treatment, have been used to inhibit PPO activity (Montero, Ávalos, & Pérez-Mateos, 2001; Ozoglu & Bayindirli, 2002; Weemaes, Ludikhuyze, Broeck, Hendrickx, & Tobback, 1998a; Weemaes, Ludikhuyze, Broeck & Hendrickx, 1998b). We studied pressure and temperature resistance of PPO in cloudy apple juice during $SCCO_2$ treatment. Moreover, a conventional first-order reaction was employed in the present study, similar to thermal processing and ultra high pressure (Balaban et al., 1991; Basak & Ramaswamy, 1996; Weemaes, Ludikhuyze, Broeck & Hendrickx, 1998c).

The purpose of this paper was to determine the effect of $SCCO_2$ treatment conditions, such as time, temperature and pressure, on the inactivation of PPO in cloudy apple juice, and analyze the inactivation kinetics of PPO.

2. Materials and methods

2.1. Materials

2.1.1. Preparation of cloudy apple juice

Apples (Fuji) were purchased from a local market and processed at commercial maturity. The fruits were cleaned, cored and juiced with a juice extractor (Ouke, ZHJ-308A1, Fushan Ouke Electric Appliance Co., China). To avoid undesirable enzymatic browning during the processing, 0.12% of L-ascorbic acid (Beijing Chemicals Co., Beijing, China), as anti-browning, agent, was added to the apple slices before the juice press. Then the apple juice was filtered through a 4-layer cheese cloth and stored at −18 °C in darkness. The initial pH of the apple juice was 3.95. Samples were thawed at ambient temperature before each experiment.

2.1.2. Reagents

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

2.1.3. Carbon dioxide

The purity of CO_2 was 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).

2.2. $SCCO_2$ treatment system

The schematic diagram of the system used for $SCCO_2$ treatment is shown in Fig. 1. This system consisted of a 200 ml stainless steel pressure vessel, temperature controllers, pressure gauges, 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 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 (8 MPa) was chosen slightly above the critical pressure for CO_2 (7.36 MPa) and the maximum pressure (30 MPa) was slightly less than the operating limit of the equipment (35 MPa). The applied temperature in the study ranged from 35 to 55°C, and were all above the critical temperature of CO_2 ($T = 31.0$ °C). The treatment time varied from 15 to 60 min.

2.3. Polyphenol oxidase activity measurement

Enzymatic activity was assayed by the method proposed by Nguyen, Ketsa, and Doorn (2003) with some modification. The assay medium contained 0.5 ml of apple juice and 2.5 ml of substrate solution, which were phosphate buffer solution (PBS, 0.05 M, pH 6.0) containing 0.05 M catechol. To the blank was added 0.5 ml of apple juice which was heated at 95 °C for 90 s to inactivate PPO completely to 2.5 ml of the same substrate solution. The mixed solutions were incubated at 30 °C for 50 min. PPO activity was determined by measuring absorbance of the mixture at 420 nm, using a UV-762 spectrophotometer (Lingguang, Shanghai, China) at ambient temperature (25 ± 1 °C). One unit of

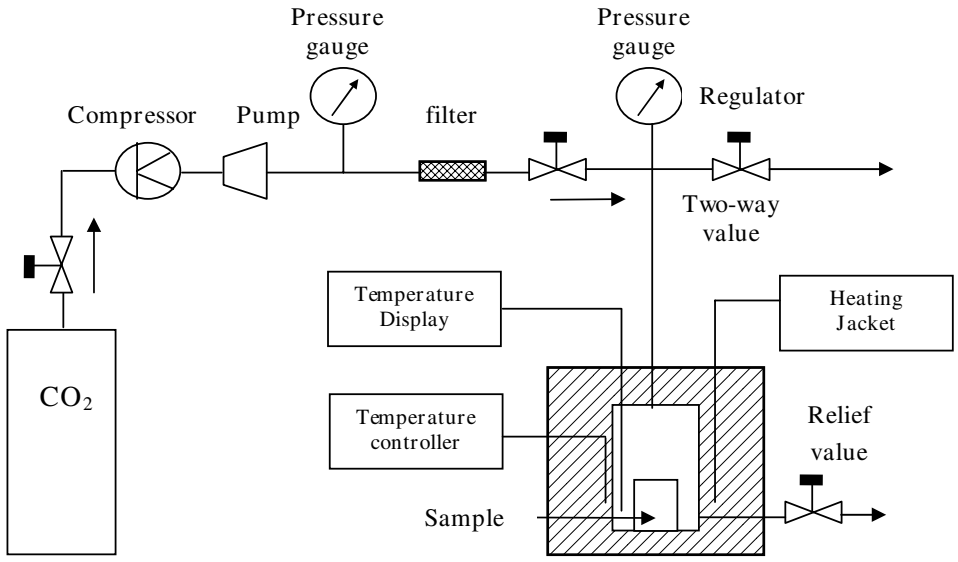

Fig. 1. Schematic diagram of supercritical carbon dioxide processing equipment.

PPO activity was defined as the change in absorbance at 420 nm/min and and per millilitre of apple juice. The relative activities of PPO were obtained with the following formula:

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

2.4. Thermal inactivation of PPO

To examine the effect of temperature alone on the PPO activity in the cloudy apple juice, thermal treatments of the apple juice were conducted at 35, 45 and 55 °C for 15, 30, 45 and 60 min, respectively. Juice (5 ml) was placed in a 18×180 mm glass tube (Beijing Bomex company, Beijing, China) and heated in a water bath at the above temperatures. The heating time was recorded after the temperature of the apple juice reached the desired temperatures. After the treatment, the tube was removed and cooled in an ice bath until used.

2.5. $SCCO_2$ inactivation of PPO in cloudy apple juice

For each experiment, 3 ml of apple juice were placed in a 10 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 pressurized by $SCCO_2$. To pressurize, CO_2 was fed over 5–10 min until the pressure reached the experimental level. The increase in the temperature in the vessel was about 2–3 °C during pressure building up and returned to the experimental level when the pressure reached the experimental level. The sample was held at constant pressure and temperature during the treatment. At the end of the $SCCO_2$ treatment, the vessel was slowly depressurized over a period of 15 min. The decrease in the temperature was about 4–5 °C during pressure release. After treatment, the apple juice was removed and immediately cooled in an ice bath. Following equilibration to ambient temperature, the residual activity and pH value were determined.

The pH values of samples before and after the $SCCO_2$ treatment were monitored by using a digital pH meter (Thermo Orion 555A, USA) equipped with a microelectrode (Thermo Orion ROSS 9103BN, USA).

2.6. Data analysis

The inactivation kinetics of PPO was analyzed by using a conventional first-order reaction (Basak & Ramaswamy, 1996):

$$\log\left[\frac{A_t}{A_0}\right] = \left[\frac{k}{2.303}\right]t \quad (1)$$

$$D = \frac{2.303}{k} \quad (2)$$

where A_t is residual enzyme activity at time t; A_0 is the initial enzyme activity and k is the reaction rate constant (min^{-1}) at given pressure and temperature. The value of k was obtained from the regression of $\log [A_t/A_0]$ versus time as $-$slope/2.303. D is the decimal reduction time, which is the treatment time needed for 90% inactivation of initial activity at a given pressure.

The pressure and temperature increase needed for a 90% reduction of the D value are reflected by Z_P (MPa) and Z_T (°C), respectively. Mathematically, they followed the equations:

$$\log\left[\frac{D_1}{D_2}\right] = \frac{P_2 - P_1}{Z_P} \quad (3)$$

$$\log\left[\frac{D_1}{D_2}\right] = \frac{T_2 - T_1}{Z_T} \quad (4)$$

The pressure- and temperature-dependence of k can be expressed by the activation volume (V_a, cm^3/mol) and activation energy (E_a, kJ/mol), as can be seen in the Eyring (5) and Arrhenius (6) equations, repectively (Balaban et al., 1991; Weemaes, Ludikhuyze, Broeck & Hendrickx, 1998c):

$$\ln\left[\frac{k_1}{k_2}\right] = \frac{V_a}{RT}[P_2 - P_1] \quad (5)$$

$$\ln\left[\frac{k_1}{k_2}\right] = \frac{E_a}{R}\left[\frac{1}{T_2} - \frac{1}{T_1}\right] \quad (6)$$

where P_2 and P_1, T_2 and T_1 are pressures and temperatures corresponding to the decimal reduction times D_1 and D_2 or constant k_1 and k_2, respectively. R is the gas constant; T is the absolute temperature (K). The values of Z_P and Z_T are obtained as the negative reciprocal slope of the regression line representing $\log D$ versus P and T relationship, respectively. E_a and V_a are estimated from linear regression of $\ln k$ versus $(1/T)$ and P, respectively.

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

3. Results and discussion

3.1. Influence of thermal treatment on PPO activity under atmospheric conditions

As shown in Table 1, different temperatures (from 35 up to 55 °C) had various effects on the inactivation of PPO in cloudy apple juice under atmospheric condition. As the temperature and time increased, the activity of PPO was decreased significantly ($P < 0.05$) apart from the treatments at 35 °C for 15 min and 30 min The maximum reduction of PPO activity was 27.9% at 55 °C for 60 min. For all enzymes studied, thermal inactivation could be described by a first-order reaction (Weemaes et al., 1998a). Dimick, Ponting, and Makower (1951) also confirmed a first-order reaction of PPO in apple by heating. The data in Table 1 were fitted to the Eq. (1), the D value of PPO in cloudy apple juice in the study were 2500, 625, 455 min at 35, 45 and 55 °C by the Eq. (2), respectively. Its E_a (activation energy) and Z_T (temperature sensitivity parameter) were 72.0 kJ/mol and 27.0 °C. Weemaes et al. (1998a) observed that two log-linear in the temperature occurred when the temperature was split into two temperature domains (⩽72.5 °C and ⩾72.5 °C) for PPO extracted from apples subjected to thermal inactivation, and its E_a and Z_T value were 111 kJ/mol and 20.1 °C when temperature was less than 72.5 °C. Our observation in the study was not in agreement with the Weemaes et al.'s report (1998a) the discrepancy maybe results from that extracted and purified PPO in Weemaes et al., 1998a study was different with crude PPO in apple juice in our experiment.

Table 1
Thermal treatment of PPO residual activity (%) in cloudy apple juice under atmospheric conditions

Treatment time (min)	35	45	55
15	99.04 ± 3.32[a]	90.57 ± 2.01	83.99 ± 1.69
30	97.02 ± 1.58[a]	87.70 ± 2.35	81.34 ± 3.34
45	96.09 ± 2.18	82.05 ± 3.02	75.37 ± 1.85
60	94.40 ± 4.36	79.75 ± 1.58	72.15 ± 2.79

[a] Mean with letter do not show significant difference at $P > 0.05$.

3.2. Influence of pressure on PPO inactivation during $SCCO_2$ treatment

Fig. 2 shows the semi-logarithmic plots of PPO residual activity, $\log(A_t/A_0 \times 100)$, versus treatment time under various pressures at 55 °C, all the plots fitted a first-order reaction. The regression coefficients R^2 (in Table 2) were greater than 0.966 ($P < 0.05$), generally indicating a good linear fit. This result confirmed the inactivation of PPO subjected to $SCCO_2$ treatment conformed to the first-order reaction. The kinetic parameters k and D value of PPO inactivation, which were computed by Eqs. (1) and (2), are tabulated in Table 2, they were closely related to the pressure levels. Higher pressures resulted in lower D values (higher k), the D value of $SCCO_2$-treated PPO was minimized to 145 min at 30 MPa and 55 °C while the D value was 455 min under atmospheric condition at 55 °C. The maximum reduction of PPO activity rose above 60% at 30 MPa and 55 °C for 60 min, which was higher than that under atmospheric conditions at 55 °C, indicating that the combined effects of pressure, temperature and time occurred after $SCCO_2$ treatment. Furthermore, the D value

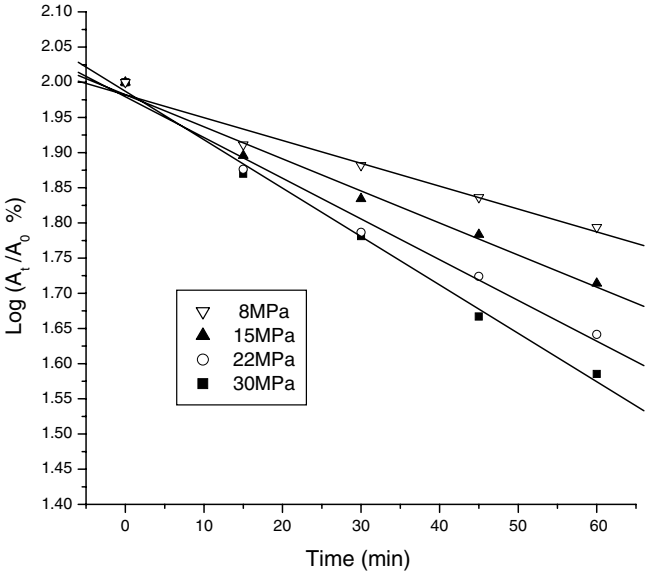

Fig. 2. Pressure inactivation kinetics of PPO at 55 °C and different pressures.

Table 2
Kinetic parameters for inactivation of PPO in cloudy apple juice at different pressures and temperatures

Temperature (°C)	Pressure (MPa)	k value ($\times 10^{-2}$ min^{-1})	D value (min)	R^2 ($P \leqslant 0.05$)
35	30	0.45	222.22	0.959
45	30	0.60	166.67	0.992
55	30	1.59	144.93	0.994
55	22	1.34	172.41	0.985
55	15	1.06	217.39	0.982
55	8	0.74	312.50	0.966

of 145 min for PPO in cloudy apple juice was far longer than the D value of 10 min for pectin–esterase in orange juice inactivated with $SCCO_2$ at 31 MPa and 60 °C (Balaban et al., 1991), suggesting that PPO in cloudy apple juice was more resistant to $SCCO_2$ treatment than PE in orange juice.

The Z_P of 66.7 MPa and V_a of −94.3 cm^3/mol at 55 °C, which were calculated by Eqs. (3) and (6), are shown in Fig. 3a and b. PPO was a single polypeptide enzyme (Martinez & Whitaker, 1995). The activation volume V_a, of −94.3 cm^3/mol, for pressure inactivation of PPO in cloudy apple juice is greater, since activation volumes are usually in the order of −30 to −80 cm^3/mol for monomeric proteins (Silva & Weber, 1993). Weemaes, Ludikhuyze, Broeck and Hendrickx (1998b) obtained Z_P of 160 MPa and V_a of −35.6 cm^3/mol of PPO extracted from apple during ultra high pressure treatment at 25 °C, which is greater than our observation, indicating that the activity of PPO is more susceptible to the variation of pressure in $SCCO_2$ treatment than in ultra high pressure treatment.

3.3. Influence of temperature on PPO inactivation during $SCCO_2$ treatment

The effect of temperature on PPO inactivation is illustrated in Fig. 4, which shows semi-logarithmic plots of residual activity versus treatment time at three temperature levels (35 °C, 45 °C and 55 °C) at 30 MPa. All three curves also fitted first-order reaction. The kinetics parameters k and D value of PPO inactivation at 30 MPa and various

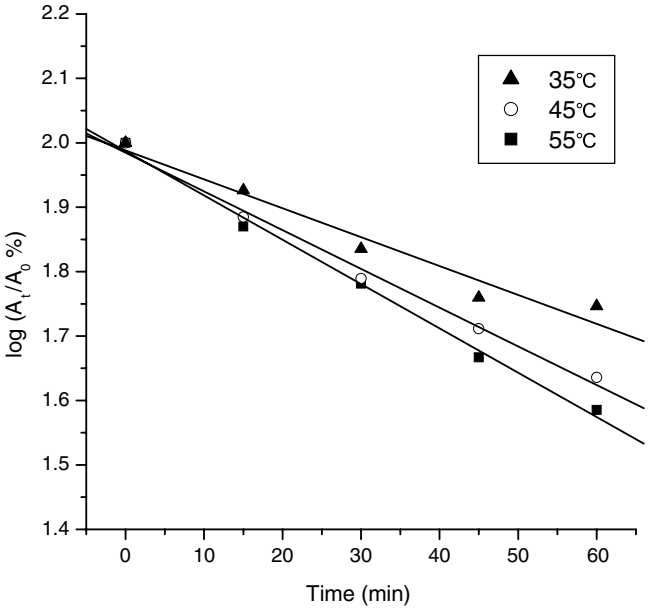

Fig. 4. Thermal inactivation kinetics of PPO at 30 MPa and different temperatures.

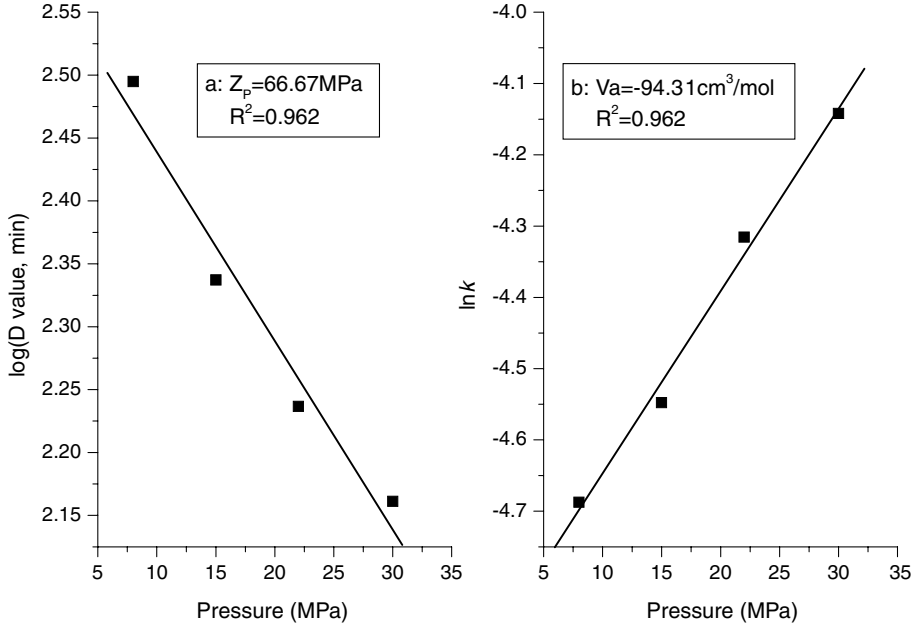

Fig. 3. Z_P value and V_a value for pressure inactivation of PPO at 55 °C during $SCCO_2$ treatment.

temperatures are also tabulated in Table 2. The regression coefficients R^2 (in Table 2) were greater than 0.959 ($P < 0.05$), indicating a good linear fit. This result further confirmed that the inactivation of PPO subjected to $SCCO_2$ treatment, followed a first-order reaction. It can be seen that increase in temperature at 30 MPa resulted in an increase of the inactivation rate constant k and a decrease of the D value.

The Z_T and E_a for PPO inactivation at 30 MPa and various temperatures, which were calculated by Eqs. (4) and (5), are displayed in Fig. 5a and b. Compared with the thermal inactivation at atmospheric pressure, the activation energy E_a was significantly decreased from 72.0 to 18.0 kJ/mol while the Z_T value increased from 27.0 to 108 °C. This suggested that increasing the pressure would increase the rate of inactivation of PPO. Such decrease of E_a and increase of Z_T with pressure were also reported by previous researchers (Weemaes, Ludikhuyze, Broeck & Hendrickx, 1998b, 1998c). Balaban (1991) observed that the activation energy of pectin-esterase in orange juice at 31 MPa decreased to 97.4 kJ/mol during $SCCO_2$ treatment while it was 167 kJ/mol at atmospheric pressure. Furthermore, Weemaes, Ludikhuyze, Broeck and Hendrickx (1998c) found that the activation energy, E_a, decreased exponentially with pressure during the combined pressure–temperature inactivation of PPO extracted from avocado.

3.4. Influence of pH on PPO inactivation during $SCCO_2$ treatment

It is difficult to monitor the pH of samples during $SCCO_2$ treatment, we only measured the pH of apple juice before and immediately after $SCCO_2$ treatment at different pressure levels and 55 °C (in Table 3). Changes in pH before and immediately after $SCCO_2$ treatment were significant ($P < 0.05$). The pH of apple juice treated by $SCCO_2$ had a maximum drop from the initial 3.95 to 3.68, less than 0.30 units. Practically, the pH change during the $SCCO_2$ treatment should be greater than 0.3 units. Balaban (1991) observed that the pH of orange juice at 31 MPa during the $SCCO_2$ treatment was lowered by about 0.7 pH units, but the pH increased and returned to its original value when pressure was released and CO_2 separated from samples. Balaban (1991) concluded that drop of pH alone was not adequate to inactivate PE in the juice. Zemel, Sims, Marshall, and Balaban (1990) showed that pH must be lowered from 3.90 to 2.50 for substantial inactivation of apple PPO. Therefore, combined effects of pressure, temperature, pH reduction, and time are suggested as the cause of PPO inactivation.

Table 3
pH Change of cloudy apple juice after $SCCO_2$ treatment at 55 °C (the initial pH of apple juice is 3.95 at 25 °C)

Treatment time (min)	8 MPa	15 MPa	22 MPa	30 MPa
15	3.74 ± 0.03	3.72 ± 0.05	3.71 ± 0.03	3.70 ± 0.03
30	3.69 ± 0.04	3.70 ± 0.03	3.69 ± 0.02	3.68 ± 0.04
45	3.69 ± 0.06	3.66 ± 0.02	3.66 ± 0.04	3.67 ± 0.05
60	3.68 ± 0.02	3.68 ± 0.04	3.66 ± 0.03	3.66 ± 0.01

The inactivation of PPO in cloudy apple juice exposed to $SCCO_2$ treatment may be due to its conformational change. Chen et al. (1992)reported that high pressure CO_2 caused conformational changes in the secondary

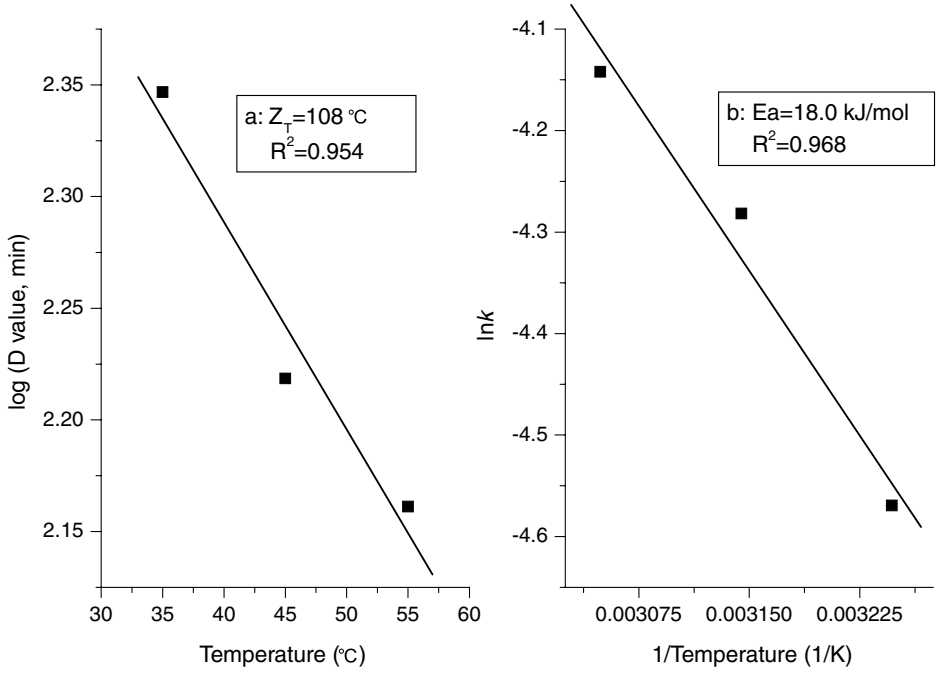

Fig. 5. Z_T value and E_a value for thermal inactivation of PPO at 30 MPa during $SCCO_2$ treatment.

structure of polyphenol oxidase (PPO). Our previous experiment also confirmed the conformation change of horseradish peroxidase in acetic buffer solution after $SCCO_2$ treatment (Gui et al., 2006).

4. Conclusions

The inactivation of PPO in cloudy apple juice exposed to $SCCO_2$ treatment followed first-order reaction kinetics. The maximum reduction of PPO activity reached more than 60% at 30 MPa and 55 °C for 60 min the D value was closely related to the pressure and temperature. Higher pressures or higher temperatures resulted in lower D values (higher k), the D value of PPO was minimized to 145 min treated at 30 MPa and 55 °C. Pressure and temperature sensitivity of kinetic parameters were determined. Z_P at 55 °C was 66.67 MPa and Z_T at 30 MPa was 107.53 °C. The activation energy E_a was reduced significantly from 72.0 to 18 kJ /mol when the pressure was increased from atmospheric pressure treatment to 30 MPa during $SCCO_2$ treatment. $SCCO_2$ treatment could effectively inactivate PPO in cloudy juice with higher pressures, higher temperatures, and longer treatment time, resulting in more inactivation.

Acknowledgments

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