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Inactivation of peroxidase and polyphenol oxidase in red beet (*Beta vulgaris* L.) extract with continuous high pressure carbon dioxide

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ABSTRACT

The inactivation of peroxidase (POD) and polyphenol oxidase (PPO) in red beet extract (RBE) with continuous high pressure carbon dioxide (HPCD) was investigated. HPCD treatment at 7.5 MPa (55 °C, 30 min) resulted in a reduction of their activities by approximately 73% and 93%, respectively. Compared with thermal treatment, continuous HPCD treatment reduced the decimal reduction time (*D*) of POD and PPO from 555.6 min to 55.9 min and 161.3 min to 32.1 min, respectively. The inactivation process could be described by first-order kinetics ($r^2 > 0.70$, $p < 0.05$); *D* values declined when temperature increased and continuous HPCD at 7.5 MPa and 55 °C resulted in the highest reaction rate constant (*k* value; smallest *D* value). The activation energy of the inactivation was reduced by HPCD treatment from 92.5 kJ/mol to 69.8 kJ/mol and 57.1 kJ/mol to 49.5 kJ/mol for POD and PPO, respectively. Continuous HPCD treatment had little effect on the antioxidant capacities of RBE samples.

1. Introduction

Enzymes and microorganisms in various foods cause quality deterioration and bacterial spoilage during storage and distribution. In the food industry, heat treatment has generally been used for microorganism sterilisation and enzyme inactivation. However, it is commonly known that heat treatment impacts on product quality, including flavour, texture and nutrients, such as vitamins, phenolic compounds, etc. Therefore, non-thermal techniques have been desired and encouraged, and researchers from all over the world have tried to establish new techniques for microorganism sterilisation and enzyme inactivation, such as hydrostatic pressure, ozone, ethylene oxide exposure, γ-irradiation, and ultraviolet radiation (De Ancos, Cano, Hernandez, & Monreal, 1999; López et al., 1994; Yen & Lin, 1996; Yoshimura et al., 2001).

High pressure carbon dioxide (HPCD) treatment is one of the non-thermal methods that has been the subject of intense scrutiny for potential applications in a number of food processing fields, including the inactivation of those microorganisms and enzymes that can lead to unpleasant changes and degrade the quality of food products during storage (Damar & Balaban, 2006). Moreover, it has been proved that microorganisms and enzymes could be effectively inactivated by HPCD either in a batch or a dynamic system.

Most research has focused on batch treatments; Balaban et al. (1991) used supercritical carbon dioxide to inactivate pectinesterase in orange juice, and Chen, Balaban, Wei, Marshall, and Hsu (1992) tried to inactivate polyphenol oxidase (PPO) with high pressure carbon dioxide. Erkment (2000a, 2000b, 2000c, 2000d, 2001) reported the inactivation of *Escherichia coli*, *Listeria monocytogenes*, *Brochothrix thermosphacta*, *Salmonella typhimurium* and *Enterococcus faecalis* in broth and food materials by HPCD. Gui et al. (2006, 2007) found that supercritical carbon dioxide could effectively inactivate horseradish peroxidase (POD) in an aqueous solution and PPO in apple juice. Lipase, glucoamylase and pectinesterase were also efficiently inactivated by HPCD (Ishikawa, Shimoda, Kawano, & Osajima, 1995; Ishikawa, Shimoda, Yonekura, & Osajima, 1996). HPCD has been applied to batch treatment for the inactivation of peroxidase and polyphenol oxidase in red beet (*Beta vulgaris* L.) extract under low-to-moderate temperatures and pressures (Liu et al., 2008a).

However, the possibility of continuous HPCD treatment is of the most paramount importance to the success of this technology in industrial application; unfortunately, this has not been extensively studied. Yoshimura et al. (2001, 2002) reported that continuous microbubbles of supercritical CO_2 were highly effective for the inactivation of α-amylase and acid protease in either deionised water or a buffered solution. Kincal et al. (2005) applied a continuous HPCD system for microbial reduction in orange juice. The lack of adequate production procedure represents a big obstacle to the development of this technology with industrial applications in mind, since their limits and potentials remain undiscovered; in particular, each study should be tailored to real and specific foodstuffs and it is extremely important to investigate CO_2-substrate interactions.

Polyphenol oxidase (PPO; monophenol, dihydroxy-L-phenylalanine oxygen oxidoreductase, E.C.1.14.18.1) is a copper protein

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widely distributed across the phylogenetic scale and responsible for the enzymatic browning reaction occurring during the handling, storage and processing of the damaged tissue of fresh fruits and vegetables, as well as some animal products. In plant tissues, the browning pigments lead to organoleptic and nutritional modifications, thus depreciating the quality of the food products (Gülcin, Küfrevioğlu, & Oktay, 2005). Peroxidase (POD) is a haeme protein, which is a member of the oxidoreductases [E.C.1.11.1.7] and catalyses the oxidation of a wide variety of organic and inorganic substrates in the presence of hydrogen peroxide. Peroxides are widely distributed in living organisms, including microorganisms, plants and animals. It has been reported that POD is involved in enzymatic browning, either separately from or together with polyphenol oxidase activity (Köksal & Gülcin, 2008). More precise research into the impact of continuous HPCD on PPO and POD activities is an essential step towards a more efficient control of these undesirable reactions, particularly in plant tissue products, which frequently contain residual PPO and POD activities.

In this study, red beet (*B. vulgaris* L.) extract was treated with continuous HPCD. The objective of the present study was to investigate POD and PPO inactivation efficiencies induced by continuous HPCD and make comparisons with batch treatment as well as heat treatment. Also we wanted to analyse the changes of nutrients (betalains and total phenolic concentrations) and antioxidant capacities in red beet extract after continuous HPCD treatment.

2. Materials and methods

2.1. Materials

Red beet (*B. vulgaris* L.) roots were purchased from a local market, cleaned with tap water and then homogenised in cold distilled water (1:2.5, w/w) for 2 min using a domestic blender (Philips HR2860, Zhuhai, China). The homogenate was filtered through four layers of cloth (400 mesh) to yield red beet extract (RBE), which was frozen by liquid nitrogen and stored at −20 °C in darkness until use. Samples were thawed at ambient temperature before use. Methanol was purchased from Merck (Darmstadt, Germany) and was of HPLC grade. Other chemicals used were of analytical grade, unless otherwise stated. CO_2 was of 99.9% purity and purchased from Beijing Analytical Apparatus Co. (Beijing, China).

2.2. Continuous HPCD treatment system

The continuous HPCD treatment apparatus for liquid samples was manufactured by Huali Pump Co. Ltd., (Hangzhou, China). A schematic diagram of the HPCD treatment system is shown in Fig. 1. RBE samples and liquid CO_2 were simultaneously pumped by two two-plunger type pumps though a treatment tube (3.5 cm i.d. × 150 cm long, 1442 ml) at respective flow rates, which were monitored by mass flow rate meters. The CO_2 was changed from liquid to gaseous or supercritical state by going through a helix evaporator ahead of the treatment tube. RBE samples were also pre-heated through a helix tube immersed in a water bath. The treatment tube was enveloped by an electrical heating jacket with four thermocouples attached to the tube surface to monitor and control the temperature inside the tube. Treatment time (average residence time, *RT*) was calculated from sample flow rates. The maximum operating pressure of the system was 55 MPa.

2.3. Continuous HPCD treatment of RBE

Continuous HPCD treatments were carried out with varying parameters of pressure and temperature: 4.5, 7.5, 15, 22.5, 30 MPa (55 ± 0.5 °C); 35, 45 and 55 °C (7.5 ± 0.5 MPa). The treatment tube was pre-heated to a given temperature (see above). For each pressure and temperature, the various treatment times were achieved by setting different flow rates of RBE. The RBE samples were collected at the exit of the tube, and cooled down in an ice-water bath. The samples were immediately analysed for residual POD, PPO activities, betalains, total phenolic concentrations and antioxidant capacities.

2.4. Assay of POD and PPO activities

POD and PPO activities were assayed with the methods described in our published paper (Liu et al., 2008a), which were derived from the articles of Gülcin et al. (2005), and Köksal and Gülcin (2008) with minor modifications. The residual activity (*RA*%) of POD and PPO was calculated as the activity after treatment divided by the activity before treatment.

2.5. Analysis of betalains, total phenolic concentrations in RBE and antioxidant abilities of RBE

To compare the effects of HPCD and thermal treatments on the main pigments in RBE, the samples treated by continuous HPCD (4.5, 7.5, 15, 22.5, 30 MPa and 55 °C for 30 min) and heat (95 °C for 5 min) were analysed for concentrations of betanin and isobetanin using HPLC following the method described by Liu et al. (2008a). Residual concentrations of betanin and isobetanin for each treated sample were expressed as a percentage of the original concentration (untreated sample, regarded as control).

The amount of phenolic compounds in RBE samples was determined according to a modified Folin–Ciocalteu method (Gülcin, 2005; Gülcin, Tel, & Kirecci, 2008) with gallic acid as a standard. An aliquot of diluted extract was introduced into a test tube and mixed with 1.0 ml of 1 N Folin–Ciocalteu reagent. The mixture was allowed to stand for a 2–5 min period, which was followed by the addition of 2.0 ml of 20% $NaCO_3$. After incubating for 10 min at room temperature, the mixture was centrifuged for 8 min (150*g*) and the absorbance of the supernatant was measured at 730 nm on a Lengguang 757 UV–Vis spectrophotometer (Shanghai, China). The residual concentration of total phenolic compounds for each sample was expressed as a percentage of the original concentration.

The antioxidant capacities of RBE were estimated by ABTS radical ($ABTS^{\cdot+}$) inhibition and Fe(III) reduction abilities. $ABTS^{\cdot+}$ elimination abilities were analysed according to the method described by Ak and Gülcin (2008) with minor modifications. The degree of inhibition activity was calculated using the following formula:

$$H\% = \frac{H_0 - H_t}{H_0} \times 100, \quad (1)$$

where *H*% is the inhibition ability, H_0 is the absorbance at 734 nm of a blank sample, and H_t is the absorbance of samples at 734 nm after 20 min reaction. Residual $ABTS^{\cdot+}$ inhibition abilities (*RH*-$ABTS^{\cdot+}$%) in continuous HPCD or heat-treated samples were calculated by comparing *H*% values with those in samples without any treatment, and were expressed as a percentage of the original inhibition capacity.

The ferric-reducing ability was determined using the method described by Gardner, White, McPhail, and Duthie (2000). Fe(III)-2,4,6,-tri(2-pyridyl)-*s*-triazine (TPTZ) complex is transformed to Fe(II)-TPTZ by the present of reductant. The resulting intense blue colour (typical absorbance at 593 nm) is linearly related to the amount of reductant (Benzie & Strain, 1999). The residual ferric-reducing ability (*RFRA*%) for each sample was obtained by comparing absorbance at 593 nm with that in the sample without any treatment, and was expressed as a percentage of the original ferric-reducing activity.

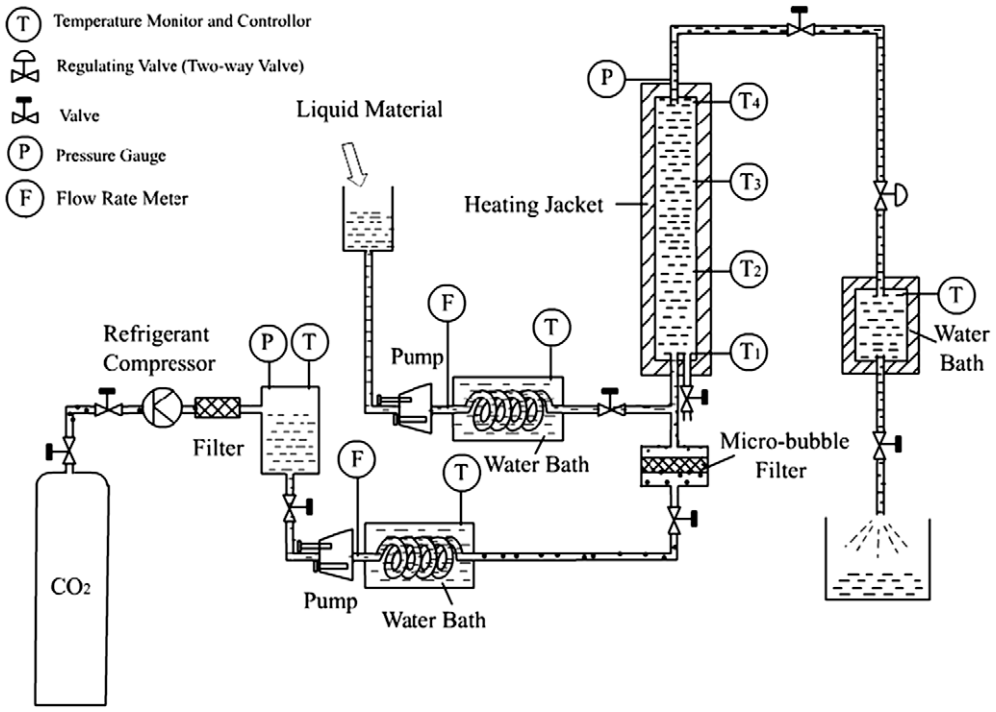

Fig. 1. Schematic diagram of the continuous high pressure carbon dioxide processing unit.

2.6. Kinetic analyses

The kinetic analyses of the inactivation of POD and PPO were followed as described in Liu et al. (2008a). Kinetic parameters were estimated using a 2-step procedure. First, natural or base-10 logarithms of the mean residual activity were regressed against treatment time at arbitrary treatment conditions, in order to calculate the inactivation rate constant ($1/k$ min) and decimal reduction time (D min value), respectively. The D value was defined as the time needed for 90% inactivation of the initial activity at a given condition. Second, Z_T (°C) values (temperature increases needed for a 90% reduction in D value) were obtained and the values of activation energy (kJ/E_a mol) were derived from the Arrhenius plot.

2.7. Statistical analysis

All experiments were conducted in duplicate and all the measurements were performed in triplicate. Data were subjected to analysis of variance (ANOVA) using the software package SPSS 12.0 for Windows (SPSS Inc., Chicago, IL). Means of treatments were separated at the 5% significance level using the LSD method.

3. Results and discussion

3.1. Inactivation kinetics for POD and PPO in RBE with continuous HPCD treatment at different temperatures

Fig. 2 shows the inactivation curves of POD and PPO in RBE when treated with continuous HPCD. With the extension of treatment time, the residual activities of POD and PPO were significantly reduced ($p < 0.05$). As expected, the inactivation of POD and PPO was strongly influenced by temperature; higher temperature resulted in more rapid inactivation. However, POD was much more heat-resistant than PPO. After 29.5 min at 55 °C, the residual activity of POD was 27.14% while the residual PPO activity was only 7.12%. At the same temperature of 55 °C, continuous HPCD could achieve more effective inactivation of POD and PPO even at

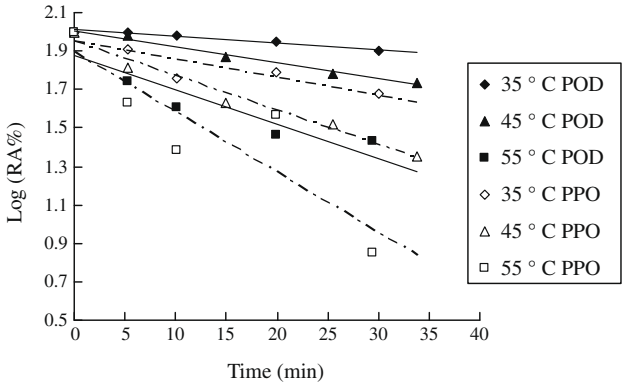

Fig. 2. Inactivation of POD and PPO in RBE by continuous HPCD treatment (7.5 MPa) at different temperatures.

lower pressure (7.5 MPa) and shorter time (29.5 min) than static HPCD treatment (37.5 MPa, 60 min) (Liu et al., 2008a). By analogy, continuous HPCD treatment is also more effective than traditional heat treatment.

Results of the regression analysis (Table 1) show that the data were well fitted to linear models ($r^2 > 0.76$, $p < 0.05$), indicating that the inactivation of POD and PPO by continuous HPCD also followed first-order kinetics. Moreover, with an increase in temperature, the D values decreased, while the k values increased. Furthermore, the D value for POD at each temperature was much higher than that for PPO, revealing that POD was still more thermally stable than PPO under continuous HPCD conditions. This agreed with the results in previous findings that POD was a more heat-stable enzyme, which required higher temperatures for complete inactivation at atmospheric conditions (Gűnes & Bayindirli, 1993).

Continuous HPCD treatments were considerably more effective than thermal treatment alone in inactivating both enzymes. For example, compared with thermal treatment at 55 °C, continuous

Table 1
Kinetic parameters for inactivation of POD and PPO in RBE by continuous HPCD treatment at 7.5 MPa compared to thermal treatment at atmospheric conditions.

	Temperature (°C)	k Value ($\times 10^{-2}$ min^{-1})	D value (min)	r^2	Z_T (°C)	E_a (kJ/mol)
Atmospheric condition Liu et al. (2008a)						
POD	35	0.05	5000	0.633	21.0 (r^2 = 0.978)	92.5 (r^2 = 0.983)
	45	0.18	1250	0.876		
	55	0.41	555.6	0.922		
PPO	35	0.37	625.0	0.967	34.0 (r^2 = 0.975)	57.1 (r^2 = 0.980)
	45	0.88	263.2	0.971		
	55	1.43	161.3	0.964		
Continuous HPCD treatment						
POD	35	0.78	294.1	0.953	28.09 (r^2 = 0.997)	69.83 (r^2 = 0.999)
	45	1.93	119.1	0.982		
	55	4.12	55.9	0.836		
PPO	35	2.21	104.2	0.813	39.06 (r^2 = 0.999)	49.53 (r^2 = 0.999)
	45	4.1	56.2	0.97		
	55	7.19	32.1	0.769		

HPCD treatment at 7.5 MPa reduced the D value of POD and PPO from 555.6 min to 55.9 min and 161.3 min to 32.1 min, respectively. Reduction in D values of similar magnitude was also observed at other temperatures for both enzymes (Table 1). Furthermore, continuous HPCD treatment at 7.5 MPa and 55 °C induced smaller D values for POD and PPO inactivation than batch treatment at 30 MPa (Liu et al., 2008a). Compared to the results of our previous research in batch inactivation with HPCD (Liu et al., 2008a), it could be found that continuous HPCD treatment was more efficient than batch treatment because continuous HPCD could induce higher k values and smaller D values.

E_a (activation energy) and Z_T are two important parameters for the inactivation of enzymes. E_a is the energy required for the inactivation to occur while Z_T is the temperature sensitivity parameter. E_a and Z_T values for PPO and POD in RBE were obtained from the Arrhenius equations and are shown in Table 1. Compared with the thermal treatment alone, HPCD treatment led to considerably smaller E_a but greater Z_T values for both POD and PPO, implying that higher pressures had generated a reduction in the activation energy for the inactivation of both enzymes and a corresponding decrease in the temperature sensitivity of the enzymes. Decreases in E_a and increases in Z_T induced by batch HPCD treatment have also been reported by Gui et al. (2007).

The E_a values for POD were greater than those for PPO while Z_T values for POD were smaller than those for PPO, irrespective of whether the samples were treated at atmospheric conditions or by HPCD. Therefore, it can be concluded that POD in RBE was more sensitive to temperature changes than PPO.

Moreover, E_a and Z_T values for both enzymes with continuous HPCD treatment were almost approaching to those with batch HPCD treatment at 30 MPa (Liu et al., 2008a). This suggests that the temperature sensitivity of POD and PPO in RBE was nearly unchanged when treated with static (at 30 MPa) and continuous (at 7.5 MPa) HPCD.

3.2. Effect of treatment pressure on the inactivation kinetics of POD and PPO in RBE

Table 2 shows the inactivating k and D values of POD and PPO in RBE with continuous HPCD treatment at different pressures (55 °C). The residual activities of both enzymes decreased significantly with the extension of treatment time ($p < 0.05$) at each pressure. The data for a given pressure could be described by a first-order kinetic model with the regression coefficient r^2 (in Table 2) greater than 0.70 ($p < 0.05$). The highest k value and smallest D value appeared at a pressure of 7.5 MPa for both enzymes with continuous HPCD treatment. This was evidently different from batch HPCD treatment which led to a higher k value and a smaller D value when treatment pressure increased from 4.5 MPa to 30 MPa (Liu et al., 2008a).

Table 2
Kinetic parameters for inactivation of POD and PPO in RBE under different continuous HPCD pressures at 55 °C.

	Pressure (MPa)	k Value ($\times 10^{-2}$ min^{-1})	D value (min)	r^2
POD	4.5	2.63	87.7	0.802
	7.5	4.12	55.9	0.836
	15	2.65	87.0	0.789
	22.5	1.84	125.0	0.811
	30	1.91	120.5	0.847
PPO	4.5	4.38	52.6	0.881
	7.5	7.19	32.1	0.769
	15	6.79	33.9	0.937
	22.5	5.25	43.9	0.709
	30	3.98	57.8	0.797

The reason for this phenomenon is not clear. At 7.5 MPa (55 °C), carbon dioxide is just over the critical point, attaining a fluid state with a density and solvent properties similar to liquids but viscosity and mass transfer characteristics of gases (Ishikawa et al., 1995). These changes in the properties of carbon dioxide around the critical point might affect its ability to interact with the molecules of the enzymes and thus the rate of enzyme denaturation. However, it is not well understood how this might occur and the subject is worthy of further investigation.

3.2.1. Effect of continuous HPCD and traditional thermal treatment on residual concentrations of betanin, isobetanin and total phenolic compounds in RBE

As shown in Fig. 3, treatment at 7.5 MPa achieved the highest residual concentration of betanin, almost unchanged. Treatments at 4.5, 15, 22.5 and 30 MPa induced similar residual concentrations of betanin. Residual concentrations of isobetanin in all the continuous HPCD-treated samples in Fig. 3 had no significant differences between each other. This phenomenon was consistent with our previous research of pigment stability under HPCD (Liu, Gao, Xu, Wang, & Yang, 2008b). Meanwhile, continuous HPCD treatments below the pressure of 22.5 MPa led to almost the same residual concentrations of total phenolic compounds, and furthermore, a little higher than in the control sample. The reason for this might be that more phenolic compounds appeared as a result of chemical degradation reactions in the RBE when treated by continuous HPCD. Traditional thermal treatment at 95 °C for 5 min approached the values for continuous HPCD treatment at pressures of 22.5 and 30 MPa. However, continuous HPCD treatment at 7.5 MPa induced

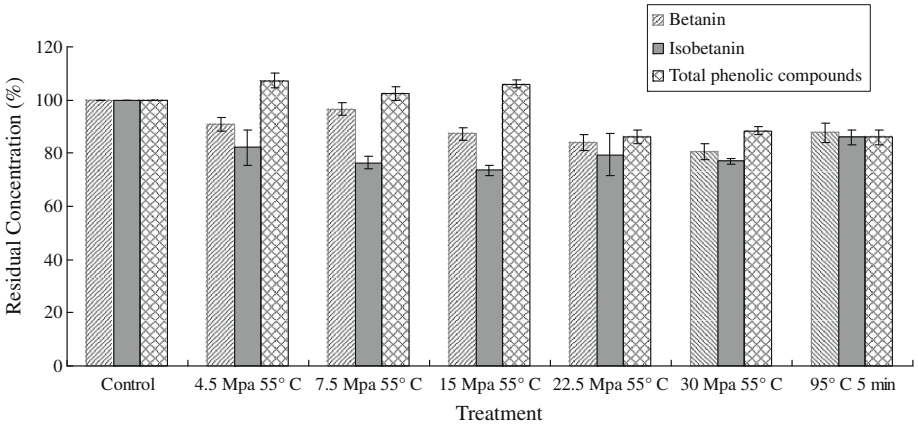

Fig. 3. Effect of continuous HPCD (treatment time: 30 min) and traditional thermal treatment (95 °C, 5 min) on concentrations of betalains and total phenolic compounds in RBE.

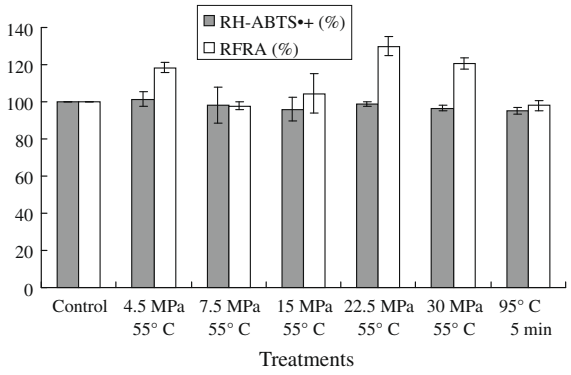

Fig. 4. Effect of continuous HPCD (treatment time: 30 min) and traditional thermal treatment (95 °C, 5 min) on antioxidant abilities of RBE.

a higher betanin but slightly lower isobetanin retention than traditional thermal treatment. When RBE sample was depressed with HPCD, much more carbon dioxide was dissolved into the aqueous extract than that under atmospheric conditions, carbonic acid formed and then resulted in the decrease of pH value. Consequently, a lower pH value might lead to isomer conversion from isobetanin to betanin. In summary, when considering the reduced loss of nutrients, continuous HPCD treatment at pressure of 7.5 MPa was better than traditional thermal treatment.

3.3. Effect of continuous HPCD and traditional thermal treatment on antioxidation activities of RBE

Fig. 4 shows the antioxidant abilities of RBE with continuous HPCD and traditional thermal treatments. As obviously shown in Fig. 4, neither continuous HPCD nor thermal treatment had a significant effect on the residual inhibition activity of $ABTS^{\cdot+}$ of RBE. Moreover, the reduction of Fe(III) activity was not alleviated with continuous HPCD and thermal treatment. On the contrary, the reduction activity of Fe(III) was even higher than the control except when treated with continuous HPCD at 7.5 MPa, 55 °C and thermal at 95 °C for 5 min. When RBE was exposed to continuous HPCD or heat, the concentration of betanin and isobetanin inevitably degraded because they are thermally-sensitive natural pigments. However, the degradation might not damage the compounds which contributed to the antioxidant properties. Therefore, the antioxidant activities of RBE samples remained unchanged when exposed to continuous HPCD or heat for a short period.

4. Conclusion

Continuous HPCD treatment has been proved effective in inactivating POD and PPO in RBE and the inactivation process followed first-order kinetics. Compared with thermal treatment, continuous HPCD treatment resulted in significant reductions in D and E_a values for the inactivation of POD and PPO in REB. Moreover, continuous HPCD treatment at a comparatively lower pressure was even more efficient than batch HPCD. Continuous HPCD treatment at 7.5 MPa and 55 °C, which led to highest k values and smallest D values for both enzymes was better than traditional thermal treatment, in the respect that it also achieved more retention of nutrients. Furthermore, both continuous HPCD and thermal treatment exhibited little effect on the antioxidant properties of RBE samples.

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