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Inactivation kinetics and secondary structural change of PEF-treated POD and PPO

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Abstract

Effects of pulsed electric fields (PEF) on the activity of peroxidase (POD) and polyphenol oxidase (PPO) in buffered solution were studied while the corresponding changes to their secondary structures was demonstrated by far-UV Circular dichroism (CD). The relative residual activity of POD and PPO decreased with the increase in electric field strength and treatment time, and PPO was more susceptible than POD to PEF treatment. The greatest reduction of the activity was achieved for POD at 25 kV/cm for 1740 μs and PPO at 25 kV/cm for 744 μs with reductions of 32.2% and 76.2%, respectively. The inactivation kinetic parameters *D*-value and Z_E value were calculated. The *D*-values of PPO were smaller than those POD at higher electric field strength, and Z_E values of POD and PPO were 36.9 and 16.2 kV/cm, respectively. The secondary structures of the two enzymes were changed following treatment by PEF. The intensity of negative peaks in the CD spectra decreased, and the CD spectra of PPO changed more significantly than that of POD; the reduction of the relative α-helix fractions for POD at 25 kV/cm for 124 μs was 22.63% while it was 50.72% for PPO at 25 kV/cm for 52 μs. The inactivation of PEF-treated POD and PPO was in close agreement with their secondary structure changes.

Keywords: Pulsed electric fields; Peroxidase; Polyphenol oxidase; Circular dichroism

1. Introduction

Pulsed electric field (PEF) technology represents a more promising alternative to traditional thermal processing such as heat pasteurization and commercial sterilization because it can better maintain the flavor, color, taste, and nutrients of foods (Zhong, Hu, Zhao, Chen, & Liao, 2004). As an emerging non-thermal food-preservation technology, PEF has been researched and developed close to the commercial stage (Barbosa-Canovas, Pierson, Zhang, & Schaffner, 2000; Min, Jin, Min, Yeom, & Zhang, 2003, 2003; Zhang, Qiu, & Sharma, 2002). Most studies of the PEF process are focused on the inactivation of microorganisms. It was shown that PEF had a significant lethal effect on microorganisms in the processing of liquid foods (Barbosa-Canovas, Gongora-Nieto, Pothakamury, & Swanson, 1999; Jeyamkondan, Jayas, & Holley, 1999; Ravishankar, Fleischman, & Balasubramaniam, 2002).

Similar to thermal processing, PEF also decreases the activity of enzymes. However, limited reports are available on the inactivation of enzymes by PEF. Moreover, the conclusions about the effects of PEF on enzyme inactivation from different research groups are inconsistent or even contradictory (Ho, Mittal, & Cross, 1997; Loey, Verachtert, & Hendrickx, 2002; Yang, Li, & Zhang, 2004). For example, 40% reduction of PPO in buffer solution was achieved at 50 kV/cm with 30 pulses using a batch treatment chamber (Ho et al., 1997),while some reports showed 97%, 70% and 62% reduction of PPO in apple, peach and pear after PEF treatment at 24 kV/cm with 300 pulses, 24 kV/cm with 400 pulses and 22 kV/cm with 300 pulses, respectively (Giner,

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Gimeno, Rosel, Canovas, & Martin-Belloso, 1999; Rauret-Arino, Barbosa-Canovas, & Martin-Belloso, 1997). The discrepancy between those investigations was mainly due to differences in PEF treatment conditions, enzyme media during processing and electrode configuration and size.

Although the kinetic models of PEF inactivation of microorganisms are established, a model of PEF-treated enzyme inactivation was not available until present (Peleg, 1995; Rodrigo, Ruíz, Barbosa-Cánovas, Martínez, & Rodrigo, 2003; Zhang, Chang, Barbosa-Cánovas, & Swanson, 1994). A conventional first-order model $\log(A/A_0) = -t/D$ is generally used to describe the kinetics of enzyme inactivated by thermal processing and ultra high pressure (Basak & Ramawamy, 1996). Therefore, this first-order model was employed in the present study, the kinetics parameters D-value and Z_E were obtained from the model.

It was observed that after PEF treatment the secondary structure of HRP and papain was changed by CD analysis, which was related to the inactivation of enzymes (Yeom, Zhang, & Dunne, 1999; Zhong et al., 2004). However, the PEF inactivation mechanism of enzymes is not completely elucidated or understood.

Peroxidase (POD, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.14.18.1) are the important enzymes in many fruits and vegetables. Their residual activity is detrimental to the quality of processed products of fruits and vegetables resulting in effects such as browning, off flavor and loss of vitamins. Therefore, the inactivation of POD and PPO in the processing of fruits and vegetables is a major quality indicator of processed fruits and vegetables.

Based on the comparison of our preliminary experiments and our results in a previous study (Zhong et al., 2004), in this work we selected the parallel electrodes because they had a homogenous electric field.

The objective of this study was to investigate and compare the inactivation kinetics and secondary structure change of POD and PPO selected as model enzymes for PEF treatment in the study.

2. Materials and methods

2.1. Materials

Peroxidase (from horseradish, 300 U/mg) was purchased from Shanghai Xueman Biotechnology Co. (Shanghai, China) and polyphenol oxidase (T7755, from mushroom, 2000 U/mg) from Sigma Co. (St. Louis, MO, USA). The molecular weight of POD and PPO was 44 and 128 kDa, respectively. Reaction substrates such as guaiacol, hydrogen peroxide and catechol were obtained from Beijing Chemicals Co. (Beijing, China). All chemicals used in the investigation were of analytical grade.

2.2. PEF treatment system

PEF treatment was performed using a laboratory scale pulse generator system (jointly designed by Tsinghua University and China Agricultural University, Beijing, China), which was the one used by Zhong et al. (2004). Co-axial electrodes were used in that study while parallel-plate electrodes were used in the present research. Fig. 1 shows a schematic diagram of the apparatus used in this study, which included a high voltage pulse generator, a high voltage pulse treatment chamber, a peristaltic pump, an asepsis cabinet and a cooling coil. Some equipment parameters were exponentially-decaying wave, 10 Hz pulse frequency, 0.029 μF capacitor, 6 ml treatment chamber, and 52.5 ml/min flow rate. The electrodes were round with a 1.38 cm radius,they were constructed of stainless steel and the gap between the electrodes was 1 cm. Pulse width was 1.4 and 0.6 μs when the treated solution was acetate buffer (POD) and phosphate buffer (PPO), respectively. A thermocouple was attached to the exit of the chamber to monitor the post-treatment temperature.The cooling coil could effectively keep the temperature of the PEF-treated buffer solution under 40 °C by submerging it into an ice-water bath. High voltage was monitored by a Trek oscilloscope (Tektronix TDS 210, Tektronix, OR).

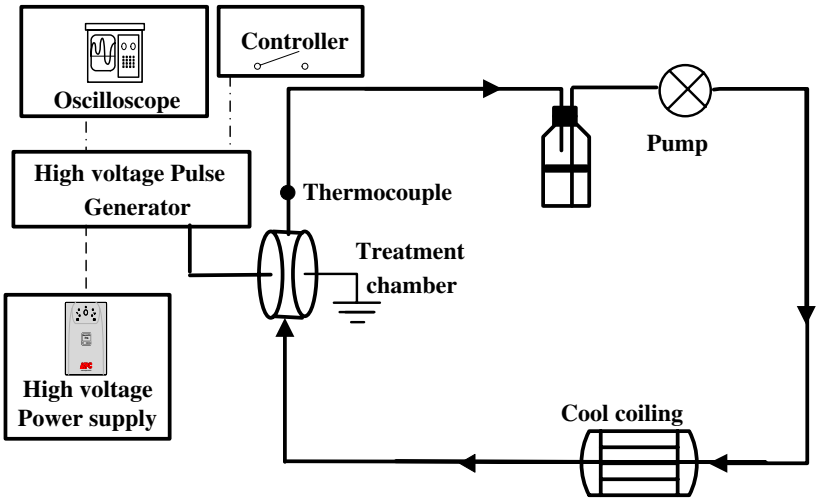

Fig. 1. Schematic diagram of the pulsed electric field equipment.

2.3. Determination of POD and PPO activity

The activity determination of POD and PPO was performed by a UV-762 spectrophotometer (Lingguang, Shanghai, China).

The POD activity was assayed by using hydrogen peroxide and guaiacol as the reaction substrates. The reaction medium contained 7 μl of 30% hydrogen peroxide and 28 μl of liquid guaiacol in 100 ml of 0.1 M acetate buffer, pH 5.6. POD concentration was 0.025 μM. The POD activity was determined by adding 0.1 ml of POD to 5.9 ml substrate solution. The mixed solution was allowed to stand for 30 min at 30 °C. The absorbance at 478 nm, due to the formation of brown guaiacol oxidation products, was measured at room temperature (25 ± 1 °C) (Adams, Brown, Ledward, & Turner, 2003).

The PPO activity was assayed with catechol as a substrate. The reaction mixture included 2 ml of 0.1 M catechol and 1 ml of 0.1 M phosphate buffer at pH 6.8 which were incubated at 37 °C. Then 2.0 ml of 0.0133 μM (3.4 U/ml) enzyme solution was added to the reaction mixture to initiate the enzyme reaction. The mixed solution was allowed to stand for 4 min at 37 °C. Absorbance at 420 nm was monitored at room temperature (25 ± 1 °C).

The specific activity and relative activity of POD and PPO were calculated with the following formulas:

Specific activity = $A_{478\ nm}$(POD) or $A_{420\ nm}$(PPO)/min/0.1 ml of undiluted enzyme buffer solution (Adams et al., 2003)

$$\text{Relative activity} = \frac{\text{Specific activity of PEF} - \text{treated POD or PPO}}{\text{Specific activity of untreated POD or PPO}}.$$

Untreated buffer solution of POD and PPO was circulated in PEF system under the same processing conditions without PEF treatment.

2.4. Inactivation kinetics of POD and PPO by PEF

The inactivation kinetics of POD and PPO were analyzed using a conventional first-order model (Basak et al., 1996)

$$\log(A/A_0) = -t/D,$$

where A is the mean residual enzyme activity at time t, A_0 the mean initial enzyme activity, t treatment time (treatment time × pulse width), μs; D the decimal reduction time, the PEF treatment time needed for 90% inactivation of initial activity at a given electric field strength, μs.

D values were obtained using procedures analogous to that employed in thermal death time studies by plotting the logarithm of A/A_0 versus time. The electric field sensitivity parameter, Z_E value was the electric field strength range between which the D value changed 10-fold. Mathematically, it followed the equation:

$$\log(D_1/D_2) = (E_2 - E_1)/Z_E,$$

where E_2 and E_1 were electric field strengths corresponding to decimal reduction times D_1 and D_2, respectively. The value of Z_E was obtained as the negative reciprocal slope of the regression line representing $\log D$ versus E relationship.

2.5. Circular dichroism (CD) analysis of POD and PPO

CD spectra were recorded with a JASCO J-720 CD spectropolarimeter (Japan Spectroscopic Company, Tokyo, Japan), using quartz cuvette of 1 mm optical path length at room temperature (25 ± 1 °C). CD spectra were scanned at the far UV range (250–200 nm) with four replicates at 50 nm/min. The CD data was expressed in terms of mean residual ellipticity, (θ), in deg cm^2 dmol^{-1}. The POD and PPO concentrations for CD analysis were 2.89 and 1.56 μM, respectively

$$\text{α-Helix relative content} = \frac{\text{content of α-helix after treatments} \times 100\%}{\text{content of α-helix before treatments}}.$$

Fraction α-helix = $-((\theta) + 4000)/29{,}000$ (Greenfield & Fasman, 1969).

2.6. Statistical analysis

Analysis of variance (ANOVA) was performed to compare the effects of electric field strength and treatment time at 5% confidence level. All experiments were repeated three times.

3. Results and discussion

3.1. Inactivation of POD and PPO by PEF

Electric field strength and treatment time (treatment time × pulse width) in the PEF treatment were the two most important factors that influenced the activity of enzymes. The effects of the electric field strength and treatment time on the activity of POD and PPO was investigated, while the temperature of buffer solution was maintained below 40 °C in all experiments. No significant change of the activity of the untreated POD and PPO occurred at 35 °C (data not shown).

The inactivation of PEF-treated POD and PPO as a function of electric field strength at a fixed treatment time is shown in Fig. 2(a) and (b). The relative residual activity of POD and PPO decreased as the electric field strength increased, indicating that the higher the electric field strength of PEF treatment, the greater was the reduction of POD and PPO residual activity. The electric field strength had significant effects on the relative residual activity of POD and PPO ($P < 0.05$). The residual activity of POD and PPO had good linear relationships with electric field strength, with regression coefficients of 0.827 and 0.933, respectively. The greatest reduction of the activity of POD was 14.3% at 25 kV/cm for 290 μs and of PPO was 16.9% at 25 kV/cm for 124 μs.

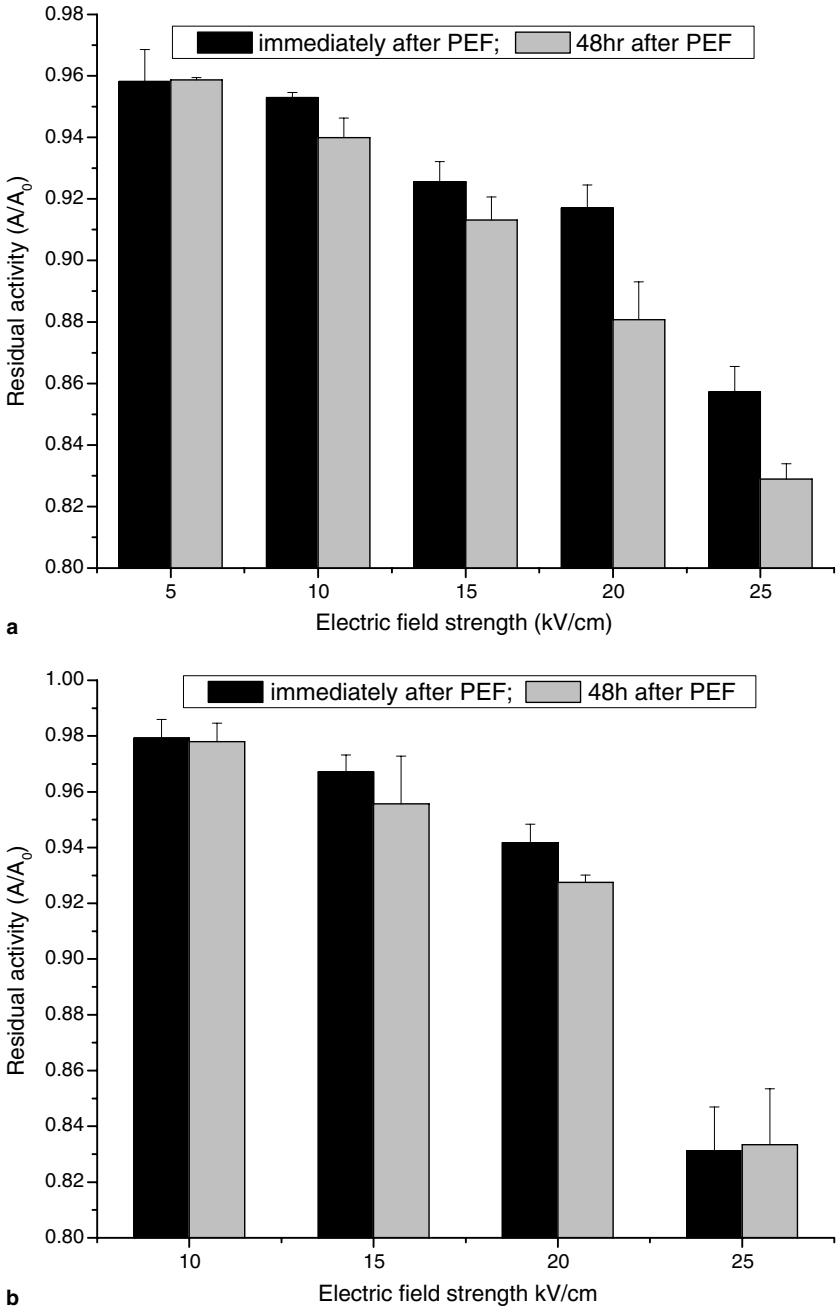

Fig. 2. (a) Inactivation of POD exposed to electric field strength varied from 5 to 25 kV/cm for 290 μs measured immediately after PEF treatment and after storage for 48 h at 4 °C. (b) Inactivation of PPO exposed to electric field strength varied from 5 to 25 kV/cm for 124 μs measured immediately after PEF treatment and after storage for 48 h at 4 °C.

Fig. 3(a) and (b) show the inactivation of PEF-treated POD and PPO as a function of treatment time at 25 kV/cm. The relative residual activity of POD and PPO gradually decreased due to the increase of treatment time, indicating that the longer the treatment time, the greater was the reduction of POD and PPO residual activity. Moreover, when the treatment time was prolonged at 25 kV/cm, POD showed a slower reduction, but PPO exhibited a sharper decrease. The treatment time had a significant influence on the relative residual activity of POD and PPO ($P < 0.05$). The residual activity of POD and PPO also had a good linear relationship with the PEF treatment time, with regression coefficients of 0.932 and 0.926, respectively. The greatest reduction of the activity of POD was 32.2% at 25 kV/cm for 1740 μs and of PPO was 76.2% at 25 kV/cm for 744 μs in the present study. Higher inactivation of PPO by PEF treatment with longer treatment time was reported, for instance, 97% in apple extract at 24.6 kV/cm for 6000 μs, 72% in pear extract at 22.3 kV/cm for 6000 μs (Giner, Gimeno, Barbosa-Canovas, & Martin, 2001), and 70% in peach extract at 24.3 kV/cm for 5000 μs (Giner et al., 2002).

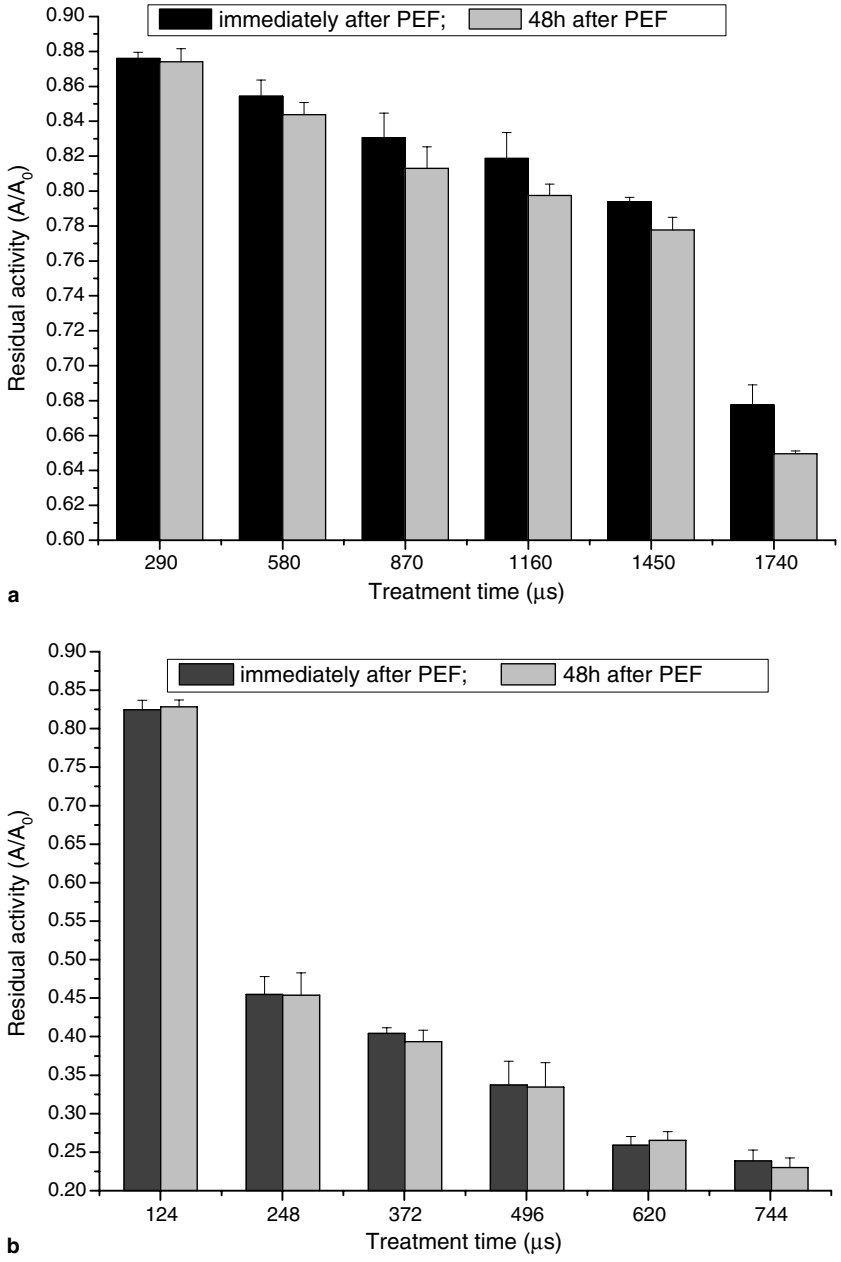

Fig. 3. (a) Inactivation of POD as a function of PEF treatment time from 290 to 1740 μs at 25 kV/cm measured immediately after PEF treatment and after storage for 48 h at 4 °C. (b) Inactivation of PPO as a function of PEF treatment time from 124 to 744 μs at 25 kV/cm measured immediately after PEF treatment and after storage for 48 h at 4 °C.

The relative residual activity of POD and PPO was also observed after storage for 48 h at 4 °C in Figs. 2(a) and (b), 3(a) and (b). As the storage time was prolonged, their relative residual activity showed a slight change, but no significant difference of POD and PPO activity was exhibited between 0 and 48 h storage at 4 °C ($P > 0.05$). This result was consistent with our previous report (Zhong et al., 2004), but it did not comply with the observation that relative activity of papain exhibited a significant decrease of activity (about 90% reduction) after storage for 48 h at 4 °C regardless of the electric field strength (Yeom et al., 1999).

3.2. D-value and Z_E value of POD and PPO

The kinetic parameter, *D*-value (treatment time needed for 90% enzyme activity reduction), was computed from $\log(A/A_0)$ versus time plots based on the above results. Fig. 4 presents *D*-values of PEF-treated POD and PPO as a function of the electric field strength and it can be seen that their *D*-values decreased steeply as the applied electric field strength increased. This further showed that higher electric field strength could result in higher inactivation level of POD and PPO. At higher than 15 kV/cm, the *D*-values of PPO were much smaller than those of POD,

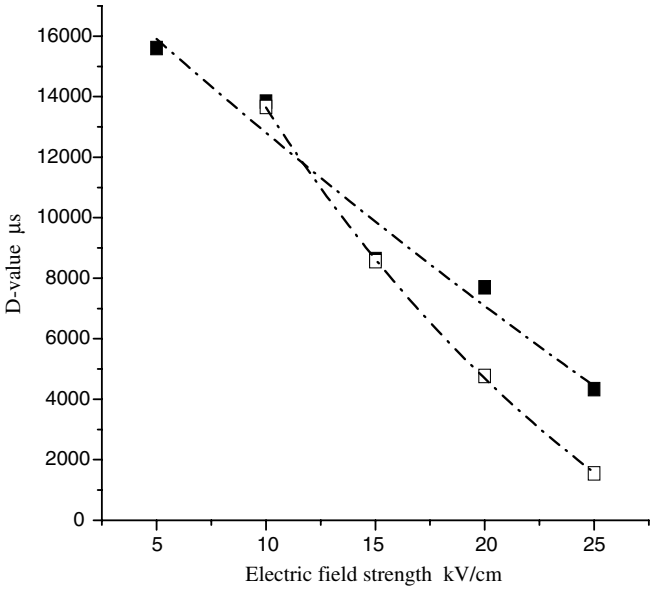

Fig. 4. Effect of applied electric field strength on the *D*-values of POD and PPO: (■) POD; (□) PPO.

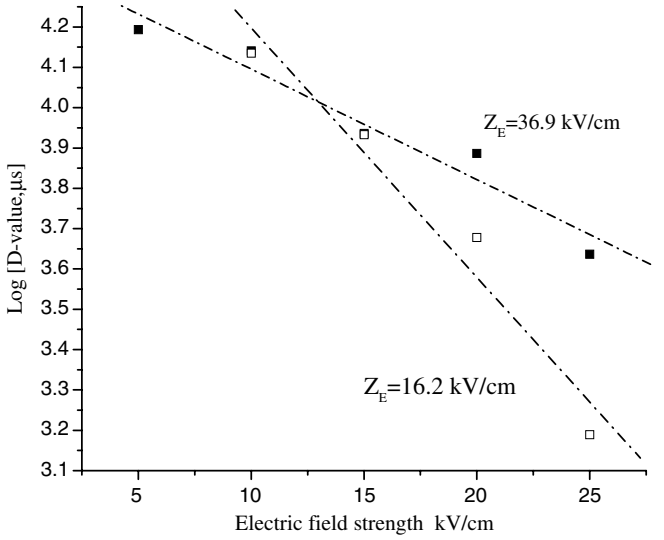

Fig. 5. Electric field sensitivity of POD and PPO inactivation rate in buffer solution (■) POD; (□) PPO.

which indicated that PEF had a greater inactivation effect on PPO than POD at higher electric field strength. According to the meaning of *D*-value, it referred to the susceptibility of one enzyme to PEF treatment, the higher the *D*-value of one enzyme was, the less its susceptibility. Therefore, it was concluded that PPO was more susceptible than POD to PEF treatment at higher electric field strength. In general, the larger an enzyme and the more complex its structure, the more susceptible it is to high temperature (Yang et al., 2004). Similarly, because the structure of PPO, which is a tetramer with a molecular weight of 128,000 is much larger and more complex than that of POD, which is a monomer with a molecular weight of 44,000, it could be reasoned that the difference in their structure caused the greater sensitivity of PPO to PEF treatment. This reasoning was further proved by the CD analysis in the following text. However, this finding was inconsistent with the study of Yang et al. (2004), who reported that the sensitivity of Pepsin (a monomer with a molecular weight of 35,500) and PPO to PEF was similar, and suggested that the mechanism involved in inactivation of enzymes by PEF may be different from that by heat.

The sensitivity of *D*-values to the electric field strength could be expressed by the parameter Z_E, which represented the electric field strength range that resulted in a 10-fold change in the *D*-values. The Z_E values were generally obtained as the negative reciprocal slopes of regression plots of logarithms of *D*-values versus electric field strength. Fig. 5 shows the electric field sensitivity of POD and PPO inactivation rate. Logarithms of *D*-values of POD and PPO had good linear relationships with the electric field strength, the regression coefficient *R* of the two lines was 0.9727 and 0.9776, respectively. Z_E values of POD and PPO obtained from the slopes were 36.9 and 16.2 kV/cm. It was concluded that inactivation change of

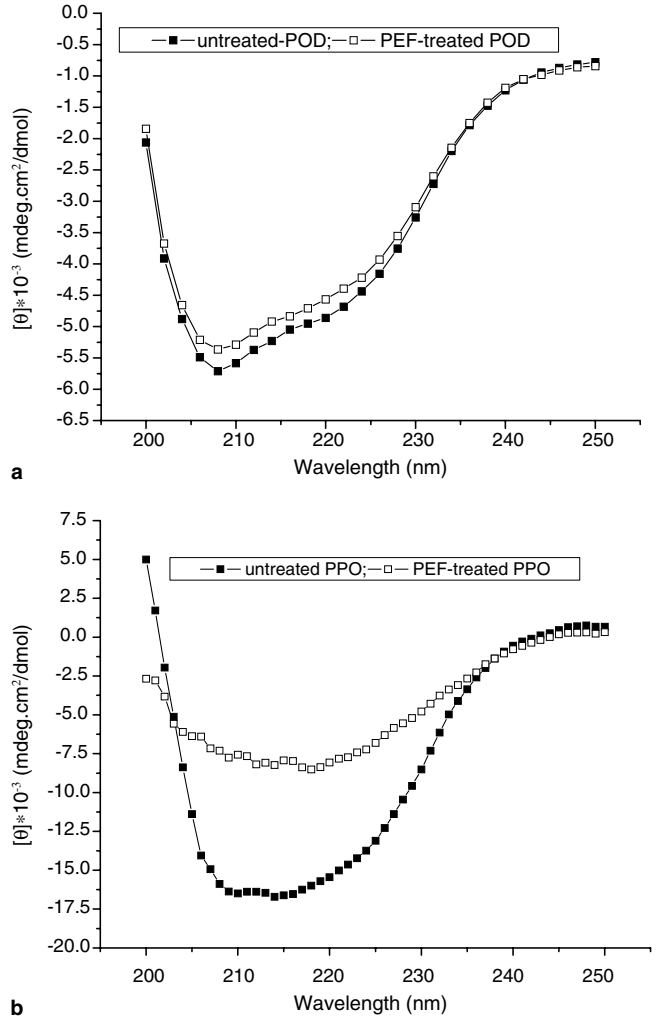

Fig. 6. (a) Far-UV CD spectra of untreated POD and PEF-treated POD at 25 kV/cm for 122 μs. POD concentration was 2.89 μM. (b) Far-UV CD spectra of untreated PPO and PEF-treated PPO at 25 kV/cm for 52 μs. PPO concentration was 1.56 μM.

POD was smaller than that of PPO due to the increase of electric field strength.

3.3. CD spectra analysis of POD and PPO by PEF

CD is a valuable spectroscopic technique for studying protein conformation in solution because many common conformational motifs containing α-helices, β-pleated sheets, poly-L-proline II-like helices and turns, have characteristic far UV (178–250 nm) CD spectra (Greenfield, 1999), and directly characterize the change of protein secondary conformation (Tang et al., 2002; Venyaminov & Yang, 1996). It is commonly known that two negative peaks at 208 and 222 nm are characteristic of the α-helix secondary conformation of proteins, and that at 214 nm is characteristic of the β-sheet secondary conformation of proteins (Chang, Wu, Venyaminov, & Yang, 1978; Myer, 1968). The far UV CD spectra of all the samples were measured immediately after PEF treatment. The CD spectra of PEF-treated POD at 25 kV/cm for 124 μs and PPO at 25 kV/cm for 52 μs are illustrated in Fig. 6(a) and (b), respectively. As shown in Fig. 6(a) and (b), it was found that the CD spectrum change of PPO was more dramatic than that of POD, testifying the above reasoning that PPO was more susceptible than POD to PEF treatment.

Fig. 6(a) showed that POD itself has double negative peaks at around 208 and 222 nm in far UV CD spectra. This observation was similar to earlier reports (Amishka Kamal & Behere, 2001; Carvalho et al., 2003; Tang et al., 2002), and their peak intensity reflected the amount of helical structure in the protein (Venyaminov & Yang, 1996). After PEF treatment the intensity of the two negative peaks in the CD spectra of POD decreased as compared with untreated POD, which indicated a loss of α-helix conformation of POD after PEF treatment. According to the equation of α-helix fraction (Greenfield & Fasman, 1969), the absolute α-helix fractions of untreated POD were 58.97%, while the absolute α-helix fraction of PEF-treated POD was 45.63%.

The secondary structure of PPO was different from that of POD. Fig. 6(b) showed that the CD spectra of PPO had two negative peaks at around 208 and 214 nm, indicating that it had β-sheets in its secondary structure besides α-helix. According to the equation of α-helix fraction (Greenfield & Fasman, 1969), the absolute α-helix fractions of untreated PPO and PEF-treated PPO were 21.0% and 10.35%, respectively. These observations of the untreated PPO absolute α-helix fraction and its secondary structure were in agreement with a previous report, in which Karbassi, Haghbeen, Saboury, Ranjbar, and Moosavi-Movahedi (2003) determined that the percentage of α-helix, β-sheet of PPO was 24.6%, 22.1% and 32.8%, respectively. After PEF treatment, not only the intensity of the two negative peaks decrease sharply but also the profile the of CD spectra of POD exhibited significant changes, indicating a dramatic secondary structure change had occurred.

After PEF-treatment, the loss of the relative α-helix fractions of POD and PPO was calculated and presented in Fig. 7. The reduction of the relative α-helix fractions for POD at 25 kV/cm for 124 μs was 22.63% while it was 50.72% for PPO at 25 kV/cm for 52 μs. Although the time of PEF treatment for PPO was shorter than that for POD at the same electric field strength, the reduction of the relative α-helix fractions for PPO was greater than that for POD, implying that the secondary structure of PPO was more destroyed than that of POD by PEF. This was bene-

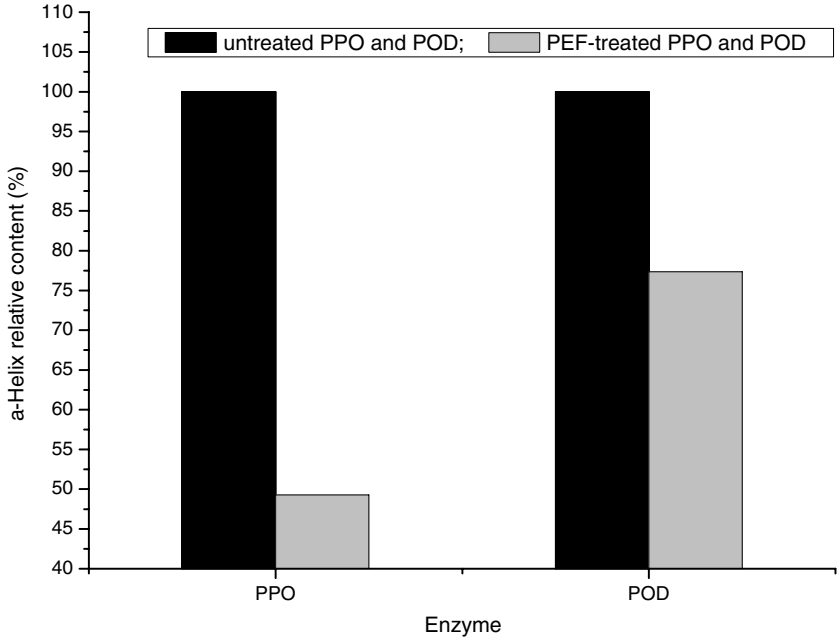

Fig. 7. α-Helix relative content of PEF-treated PPO at 25 kV/cm for 122 μs and PEF-treated POD at 25 kV/cm for 52 μs.

ficial to understand that PPO was more susceptible than POD to PEF treatment.

The inactivation and change of secondary structure of POD and PPO was observed after PEF treatment, which suggested that secondary structure change of POD and PPO was in close agreement with the inactivation of POD and PPO.

4. Conclusion

The inactivation of PEF-treated POD and PPO was observed. The relative residual activity of POD and PPO decreased with the increase in electric field strength and treatment time, and PPO was more susceptible than POD to PEF treatment. The greatest reduction of the activity of POD at 25 kV/cm for 1740 μs and PPO at 25 kV/cm for 744 μs was 32.2% and 76.2%, respectively. The inactivation kinetic parameters D-value and Z_E value were calculated. The D-values of PPO were smaller than those of POD at higher electric field strength, and the Z_E value of POD and PPO was 36.9 and 16.2 kV/cm, respectively. The secondary structure of the two enzymes treated by PEF changed as suggested by CD analysis. The intensity of negative peaks in the CD spectra decreased, and the CD spectra of PPO changed more significantly than that of POD; the reduction of the relative α-helix fractions for POD at 25 kV/cm for 124 μs was 22.63% while it was 50.72% for PPO at 25 kV/cm for 52 μs. The inactivation of PEF-treated POD and PPO was in close agreement with their secondary structure change.

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