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Inactivation and conformational change of horseradish peroxidase induced by pulsed electric field

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Abstract

Effects of pulsed electric field (PEF) on the structure and activity of horseradish peroxidase (HRP) in buffer solution were studied. The results showed that activity of HRP decreased with the increase in applied electric field strength (5–25 kV/cm) and pulse numbers (207–1242 pulses). 16.7% and 34.7% reduction of HRP relative activity was achieved immediately after PEF treatment at 25 kV/cm for 207 pulses and 22 kV/cm for 1214 pulses, respectively. The temperature of buffer solution did not increase above 40 °C during PEF treatment. The relative activity exhibited a slow reduction after 24 and 48 h of storage at 4 °C. The HRP conformation changed after PEF treatment, as suggested by CD analysis and fluorescence spectroscopy analysis. α-Helix relative content in the HRP decreased by 35.1% and 57.7% after PEF and heating at 100 °C for 5 min, respectively. The intrinsic relative fluorescence intensity (RFI) increased after PEF treatment. The inactivation of PEF-treated HRP was related to the conformational change of α-helix and the increase of RFI.

Keywords: Horseradish peroxidase; Pulsed electric field; Circular dichroism; Fluorescence spectroscopy

1. Introduction

The increasing consumer demand for high-quality minimally processed foods has stimulated the development of several non-thermal preservation technologies (Manas, Barsotti, & Cheftel, 2001). Among them, pulsed electric fields (PEF) technology represents a more promising alternative to traditional thermal processing, such as heat pasteurization and commercial sterilization, because it maintains flavour, colour, taste and nutrients of foods better. Some reports have demonstrated significant microbial inactivation upon PEF processing of aqueous solutions, including milk and fruit juices (Barbosa-Cánovas, Pothakamury, Palou, & Swanson, 1998; Barsotti & Cheftel, 1999; Barsotti, Merle, & Cheftel, 1999; Castro, Barbosa-Cánovas, & Swanson, 1993; Grah & Märkl, 1996; Jeyamkondan, Jayas, & Holley, 1999; Knorr, Geulen, Grahl, & Sitzmann, 1994; Mertens & Knorr, 1992). In comparison with the extensive research devoted to the destruction of microorganisms by PEF, there are few reports on the inactivation of enzymes by PEF (Yeom, Zhang, & Dunne, 1999).

Conventionally, enzymes in foods are inactivated by thermal processing (Ho, Mittal, & Cross, 1997). In recent years, PEF has been investigated as a potential non-thermal processing procedure to inactivate enzymes. Vega-Mercado, Powers, Barbosa-Cánovas, & Swanson (1995) reported a 90% reduction of plasmin. Vega-Mercado, Powers, Barbosa-Cánovas, Swanson, & Luedecke (1995) obtained 60% and 80% reductions of maximum proteolytic activity of protease extracted from *Pseudomonas fluorescens* dispersed in skim milk and tryptic soy broth, respectively. Ho et al. (1997) assayed eight pure enzymes in buffer solution and found that, after PEF treatment, lipase, glucose oxidase and α-amylase showed a 70–85% activity reduction; POD

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(peroxidase) and PPO (polyphenol oxidase) activities decreased by 30–40%; alkaline phosphatase was reduced by only 5%; however, lysozyme and pepsin increased their activities. Giner et al. (2000) reported a vast reduction of pectinmethylesterase (PME) from tomato (*Lycopersicon esculentum* Mill.). Most research has focussed on the inactivation of enzymes and little information is available on the mechanisms and conformation change of enzymes, due to the lack of analysis of enzyme structural data (Yeom et al., 1999).

The peroxidases (POD, E.C. 1.11.1.7) are a class of enzymes widely found in most fruits and vegetables. Their residues have a very negative effect on the quality of the processed products of fruits and vegetables. Therefore, the extent of POD inactivation is a major index of their quality. Like thermal processing, PEF also decreases the activity of POD. For example, 27% reduction of POD in soybean was achieved at 73 kV/cm using a batch treatment chamber (Ho et al., 1997). Horseradish peroxidase (HRP) is an important member of the POD family, and its structure and biochemical properties have been characterized very well (Chattopadhyay & Mazumdar, 2000; Tang et al., 2002). Therefore, HRP was selected as a model enzyme to investigate the inactivation and the conformational change of POD by PEF. In our PEF experiments, the temperature of the treated enzyme's buffer solution was controlled below 40 °C. Because POD is a heat-stable enzyme, so the experimental temperature had little effect on its activity.

2. Materials and methods

2.1. Materials

Horseradish peroxidase (abbreviated as HRP, RZ > 3300 U/mg) was purchased from Shanghai Xueman Biotechnology Co. (Shanghai, China). Reagents such as guaiacol and hydrogen were purchased from Beijing Chemicals Co. (Beijing, China). All chemicals in the investigation were of analytical grade.

2.2. PEF treatment system

PEF treatment was performed using a laboratory scale pulse generator system (designed by Tsinghua University, Beijing, China). A schematic diagram of the apparatus used in this study is shown in Fig. 1, which includes a high voltage pulse generator, a high voltage pulse treatment chamber, a peristaltic pump, a UV cabinet and a cooling coil. Some equipment parameters were exponentially-decaying wave, 1.5 μs pulse width, 10 Hz pulse frequency, 0.029 μF capacitor, 5 ml treatment chamber, and 52.5 ml/min flowing rate. The electrode configuration is co-axial type, and is shown in Fig. 2. Because the electric field strength in the treatment chamber is unevenly distributed, applied electric field strengths are averaged electric field strengths. The gap between the needle electrode (high voltage electrode) and cylinder electrode (ground electrode) was 1.0 cm. The applied electric field strength in the experiment ranged from 5–25 kV/cm, and the pulse numbers were in the 207–1449 pulses range. The high voltage was monitored by an oscilloscope (Tektronix TDS 210, Tektronix, OR). A cooling coil could effectively control the temperature of treated enzyme solution below 40 °C by submerging it in an ice-water bath. The reservoir was equipped with a UV lamp to pasteurize the cabinet.

2.3. HRP activity measurement

HRP activity was determined spectrophotometrically using a UV-762 spectrophotometer (Lingguang, Shanghai, China). Its assay was performed using hydrogen

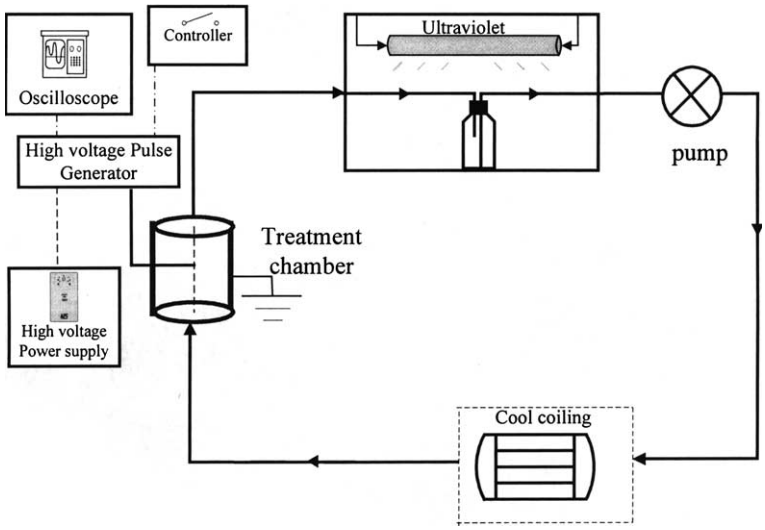

Fig. 1. Schematic diagram of the pulsed electric field equipment.

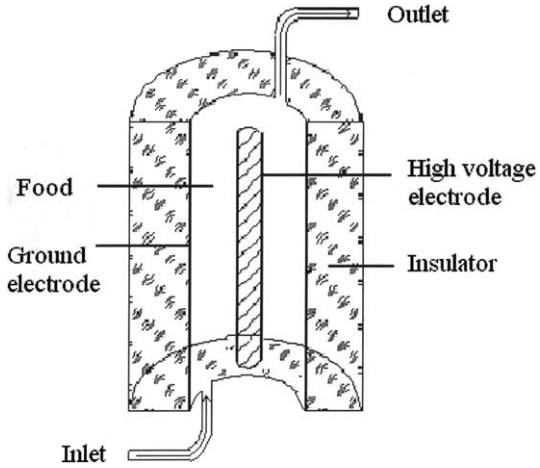

Fig. 2. Diagram of the co-axial treatment chamber.

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. HRP concentration was 0.025 μM. The HRP activity was determined by adding 0.1 ml of HRP 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). The specific activity and relative activity of HRP were calculated with the following formulas:

$$\text{Specific activity} = A_{478\ \text{nm}}/\text{min}/0.1\ \text{ml of undiluted HRP buffer solution.}$$

$$\text{Relative activity} = \frac{\text{specific activity of HRP treated with PEF} \times 100\%}{\text{specific activity of HRP before PEF}}.$$

HRP before PEF was circulated in a PEF system under the same processing conditions without PEF treatment.

2.4. Thermal inactivation of HRP

HRP (2.89 μM, 5 ml) was placed in a tube and heated in a water bath at 100 °C. The heating time was counted after the temperature of HRP reached the desired temperature (100 °C). After 5 min, the tube was removed and cooled in an ice bath until used. All of the HRP was inactivated at 100 °C for 5 min.

2.5. Circular dichroism analysis of HRP

Circular dichroism (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 room 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 2.89 μM.

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

$$\text{Fraction } \alpha\text{-helix} = -([\theta] + 4000)/29,000 \text{(Greenfield Fasman, 1969).}$$

2.6. Fluorescence spectroscopy analysis of HRP

Fluorescence spectra were measured with a HITACHI F-4500 spectrofluorometer (HITACHI, Japan), using a quartz cuvette of 1 cm optical path length at room temperature (25 ± 1 °C). The HRP concentration was 1.59 μM. The emission spectra (λ_{em}: 300–450 nm) were obtained at the maximum excitation wavelength (λ_{ex}: 234 nm) and represented the means of three scans.

$$\text{Relative fluorescence intensity} = \frac{\text{fluorescence intensity after PEF} \times 100\%}{\text{fluorescence intensity before PEF}}.$$

2.7. Statistical analysis

Analyses of variance (ANOVA) were performed to compare the effects of electric field strength and pulse numbers at 5% confidence level. All experiments were replicated three times.

3. Results and discussion

3.1. Inactivation of HRP by PEF with needle-cylinder electrode

Effects of electric field strength on HRP relative activity are shown in Fig. 3. The temperature of buffer solution was below 40 °C in all experiments. The results showed that 4.53% reduction of relative activity was achieved at 5 kV/cm for 207 pulses after 0 h (measured immediately after PEF treatment). The relative activity of HRP decreased with the increase in applied electric field strength. After PEF treatment at 25 kV/cm for 207 pulses, the reduction of relative activity reached a maximum of 16.7%. ANOVA results indicated that PEF electric field strength had significant effects on the relative activity of HRP. After storage for 24 and 48 h at 4 °C, the relative activity of HRP continued decreasing slowly, and the reductions of HRP relative activity were 17.5% and 19.3%, respectively.

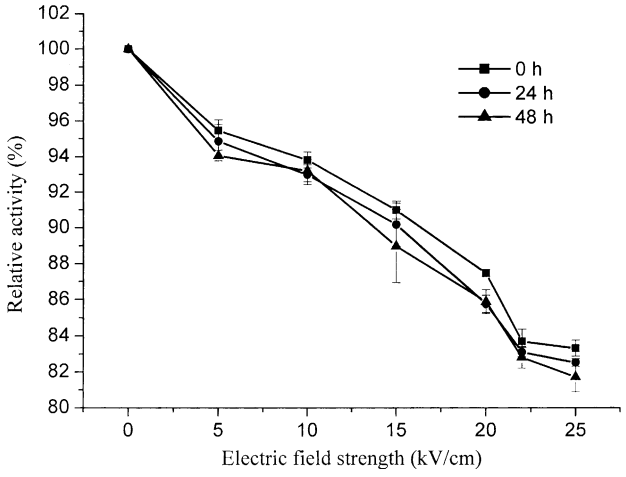

Fig. 3. Inactivation of horseradish peroxidase (HRP) exposed to electric field strength varied from 5 to 25 kV/cm with 207 pulses during storage time at 4 °C, respectively. (■), 0 h (measured immediately after PEF treatment); (●), 24 h; (▲), 48 h. The treatment temperature did not increase above 40 °C.

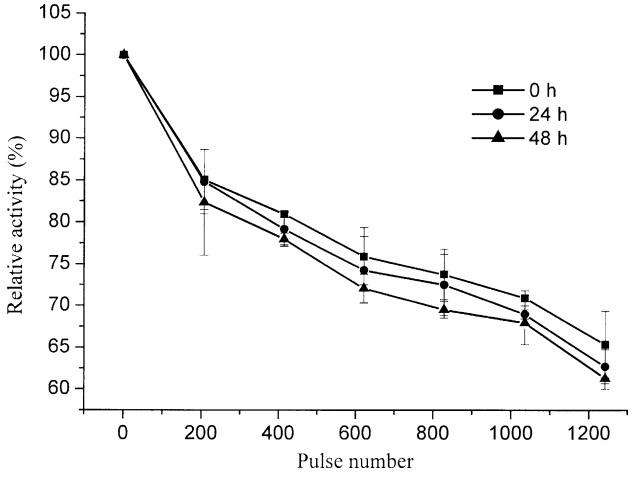

Fig. 4. Inactivation of horseradish peroxidase (HRP) exposed to electric pulse numbers varied from 207 to 1242 pulses at 22 kV/cm during storage time at 4 °C. (■), 0 h (measured immediately after PEF treatment); (●), 24 h; (▲), 48 h. The treatment temperature did not increase above 40 °C.

Besides electric field strength, pulse numbers also influenced the HRP activity. Fig. 4 shows the effects of the PEF pulse numbers on the relative activity of HRP. When electric field strength was held at 22 kV/cm, the relative activity of HRP decreased with the increase of pulse numbers from 207 to 1242 pulses. At 1242 pulses, the reduction of HRP relative activity reached a maximum of 34.7% at 0 h (measured immediately after PEF treatment) at 22 kV/cm. ANOVA results showed that pulse numbers had significant effects on the relative activity of HRP. After storage for 24 and 48 h at 4 °C, the reductions of HRP relative activity were 37.3% and 39.7%.

The activity of PEF-treated HRP showed no significant change after 0, 24 and 48 h of storage at 4 °C. This result was not consistent with the previous observation that relative activity of papain showed a significant decrease (about 90% reduction) after storage for 48 h at 4 °C, regardless of the electric field strength (Yeom et al., 1999).

3.2. Conformational change of HRP by PEF

3.2.1. CD spectra analysis

CD is a valuable spectroscopic technique for studying protein conformation in solution because many common conformational motifs containing α-helices, β-pleated sheets, and 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). The CD spectra of HRP in control and treated HRP with thermal processing and PEF are shown in Fig. 5. HPR itself has double negative peaks in far UV CD spectra at around 208 and 222 nm. This observation is similar to that obtained by Tang et al. (2002) in phosphate buffer solution. It is commonly known that two negative peaks, at 208 and 222 nm, are characteristic of the α-helix secondary conformation of protein (Chang, Wu, & Yang, 1978; Myer, 1968), and their intensity reflects the amount of helical structure in protein (Venyaminov & Yang, 1996). After treatment with PEF and thermal processing, the intensities of two negative peaks in the CD spectra of HRP decreased, indicating a loss of α-helix conformation of HRP. An estimate of the α-helix content may be made from the mean residue ellipticity

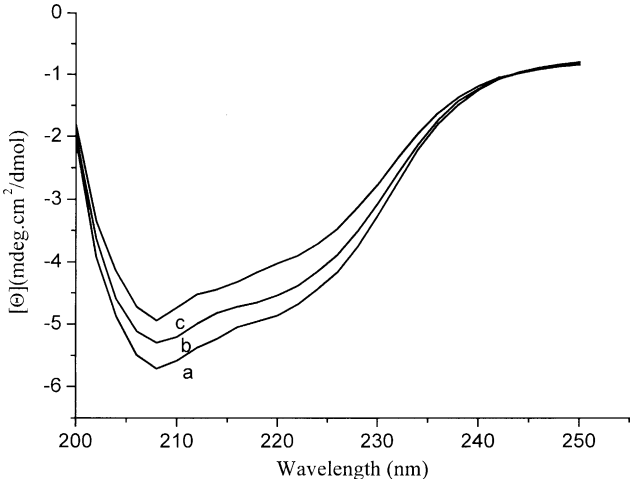

Fig. 5. Far-UV CD spectra of control HRP, PEF-treated HRP and Heat-treated HRP. HRP concentration was 2.89 μM. (a) Control; (b) PEF treatment, at 22 kV/cm with 87 pulses; the temperature did not increase above 40 °C during PEF treatment (c) thermal processing, at 100 °C for 5 min.

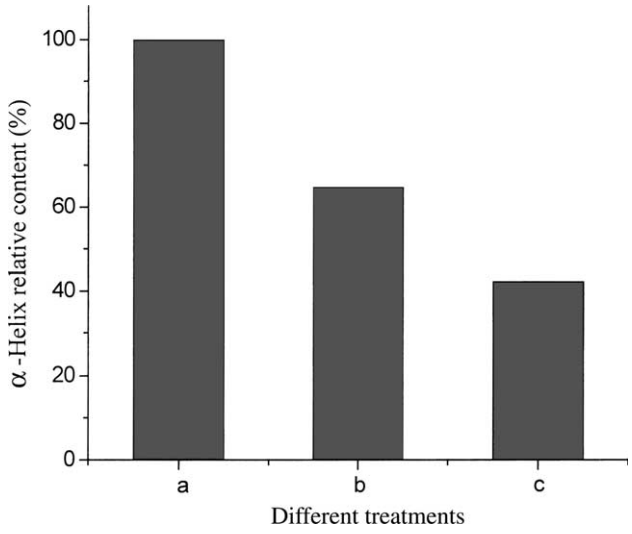

Fig. 6. α-Helix relative content of control HRP, PEF-treated HRP and heat-treated HRP at 208 nm. (a) Control; (b) PEF treatment, at 22 kV/cm for 87 pulses; (c) thermal processing, at 100 °C for 5 min. The temperature did not increase above 40 °C during PEF treatment.

at 222 nm (Morrisett, David, Pownall, & Cotto, 1973) or 208 nm (Greenfield & Fasman, 1969). Fig. 6 shows the α-helix relative content of HRP before and after PEF (measured immediately after PEF) and thermal processing at 208 nm. Two treatments decreased the α-helix content. The α-helix relative contents after PEF at 22 kV/cm with 87 pulses and thermal processing at 100 °C for 5 min, were 64.86% and 42.3%, respectively. Thus, the reduction of the α-helix relative content, caused by heating 100 °C for 5 min, was greater than that by PEF treatment.

Electric fields could cause conformational change in biopolymers and membranes (Neumann, 1986). Neumann & Katchalsky (1972) reported that electric pulses of about 20 kV/cm induced a long-lived helix-coil transition in polynucleotide helices. Loss of α-helix structure was observed in PEF-treated and thermaly processed HRP with the reduction of HRP activity. Therefore, the inactivation of HRP was related to the conformational change of α-helix induced by PEF treatment and thermal processing.

3.2.2. Fluorescence spectrum analysis

Fluorescence spectroscopy is an effective method for studying the local tertiary structure of proteins. Intrinsic fluorescence of protein is due to the existence of Trp, Tyr and Phe in protein. HRP protein has many Phe residues (Welinder, 1979), so fluorescence spectroscopy could be used to inspect local tertiary structure around the fluorophore in HRP. It can provide emission spectroscopy, excitation spectroscopy, fluorescence intensity and other physical parameters by fluorescence spectrum analysis. Therefore, the local tertiary structure of HRP protein could be obtained by determining emission spectroscopy and fluorescence intensity.

Fig. 7 shows the fluorescence emission spectra of HRP before and after PEF treatment of applied electric field strength. The pulse number was 621. Upon excitation at 234 nm, the emission maximum of control HRP was observed at 351 nm. The peak intensity in the spectra increased, accompanied by a little red shift after PEF treatment.

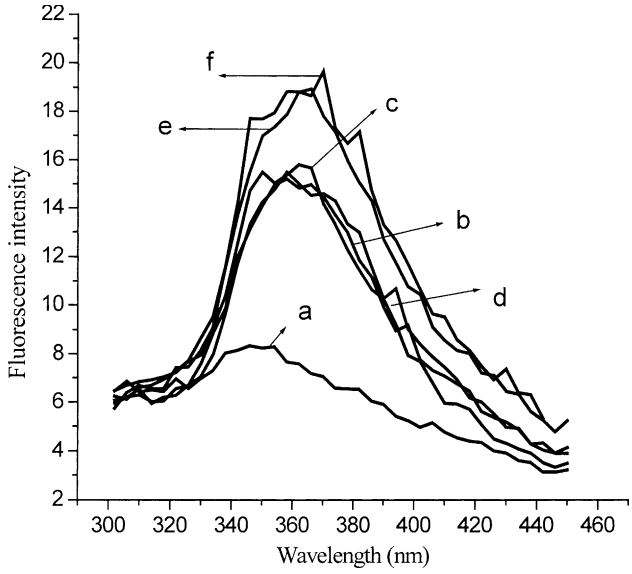

Fig. 7. Fluorescence emission spectra of HRP corresponding to applied voltages with 621 pulses. HRP concentration was 1.59 μM: (a) 0 kV/cm, (b) 5 kV/cm, (c) 10 kV/cm, (d) 15 kV/cm, (e) 20 kV/cm, (f) 25 kV/cm. The temperature did not increase above 40 °C during PEF treatment.

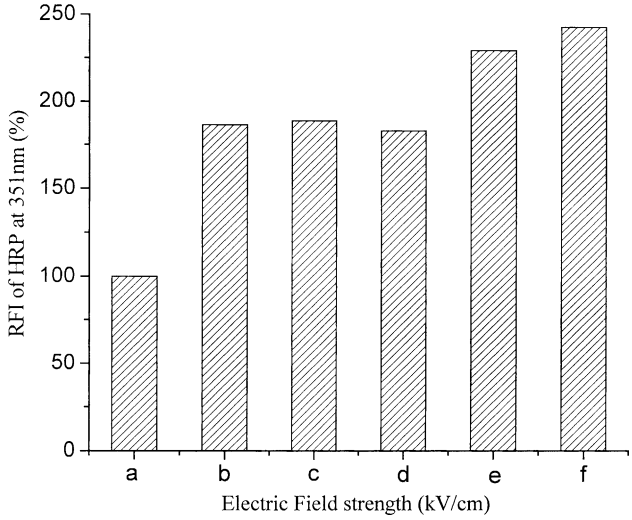

Fig. 8. Intrinsic relative fluorescence intensity (RFI) of HRP at 351 nm before and after PEF treatment. HRP concentration was 1.59 μM . (a) 0 kV/cm, (b) 5 kV/cm, (c) 10 kV/cm, (d) 15 kV/cm, (e) 20 kV/cm, (f) 25 kV/cm, 621 pulses. The temperature did not increase above 40 °C during PEF treatment.

Fig. 8 shows the change of the HRP relative fluorescence intensity (RFI) before and after PEF treatment at different electric field strengths, respectively (λ_{em}. was 351 nm). Electric field strength had a complex effect on the RFI of HRP. At low electric field strength (5 kV/cm), the RFI increased sharply and reached to 187%. However, at higher electric field strength, from 10 to 15 kV/cm, the RFI changed little and values were 188.92% and 183.29% at 10 and 15 kV/cm, respectively. Once the applied electric field strength was more than 15 kV/cm, the RFI increased again. For example, the RFI increased from 229.56% to 243.01% with increasing electric field strength from 20 to 25 kV/cm.

The change of protein intrinsic fluorescence intensity could reflect its local conformational change. After PEF treatment, activity of HRP became smaller but the fluorescence intensity of HRP became stronger. It seems that the change of RFI of HRP activity is consistent with that of applied electric field strength. Theorizing the mechanism that causes this is beyond the scope of the present study.

4. Conclusion

PEF can inactivate HRP activity and change HRP conformation. Relative activity of HRP decreased with increase of the applied electric field strength and pulse numbers. Moreover, HRP relative activity decreased slowly after storage for 24 and 48 h at 4 °C. The conformation of HRP was altered and a loss of α-helix content in HRP conformation occurred after PEF by CD analysis. The fluorescence intensity increased as the applied electric field strength increased during PEF treatment. Inactivation of HRP was related to the conformational change of α-helix and intrinsic fluorescence intensity induced by PEF.

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