Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 110 (2008) 334–343
FOOD CHEMISTRY
www.elsevier.com/locate/foodchem

The effect of pulsed electric fields on the inactivation and structure of lysozyme

Wei Zhao, Ruijin Yang *

State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi, China
School of Food Science and Technology, Jiangnan University, No. 170, Huihe Road, Wuxi 214036, China

Received 20 October 2007; received in revised form 27 December 2007; accepted 4 February 2008

Abstract

The effect of pulsed electric fields (PEF) on the activity and structure of lysozyme selected as a model enzyme was investigated. The inactivation of lysozyme in phosphate buffer was a function of electric field strength, treatment time, electrical conductivity, and enzyme concentration. No significant ($p > 0.05$) change in the activity of PEF-treated lysozyme was found after storage for 12, 24 and 48 h at 4 °C. The effect of PEF on tertiary structure of lysozyme was demonstrated by second-derivative UV spectra and intrinsic fluorescence. The results indicated that the unfolding of tertiary structure was induced by PEF treatment at 35 kV/cm for 1200 μs, and more tyrosine residues were buried inside the protein after PEF treatment, accompanied by the exposure of more tryptophan residues. CD spectra suggested that the inactivation of lysozyme by PEF was closely related to the loss of α-helix of secondary structure.

Keywords: Pulsed electric fields (PEF); Lysozyme; Tertiary structure; Secondary structure

1. Introduction

Pulsed electric fields (PEF) is a nonthermal food processing technology mainly used in liquid foods to inactivate microorganisms and enzymes selectively, retaining volatile compounds and nutrients in foods (Ayhan, Yeom, Zhang, & Min, 2001; Knorr, 1992; Mertens & Knorr, 1992). It is a potential complement to or replacement of thermal pasteurization and has undergone substantial developments near commercial application (Barbosa-Canovas, Pothakamury, Palou, & Swanson, 1998). PEF is receiving considerable attention from the food industry and has been successfully applied to a variety of liquid foods such as milk, soymilk, pea soup, liquid egg, low-acid vegetable beverage horchata and juices of orange, apple, tomato, and cranberry (Barsotti & Cheftel, 1999; Cortés, Esteve, Frígola, & Torregrosa, 2005; Elez-Martínez & Martín-Belloso, 2007; Jeyamkondan, Jayas, & Holley, 1999; Qin, Zhang, Barbosa-Canovas, Swanson, & Pedrow, 1995).

The mechanism of PEF inactivation of microorganisms has been attributed to cell membrane permeabilization and damage when the applied electric field exceeds a certain critical value (Barsotti & Cheftel, 1999; Jeyamkondan et al., 1999). Compared with the extensive studies on the inactivation of microorganisms by PEF, there are limited reports about the effects of PEF on enzymes. Most of the research work on enzyme inactivation by PEF was focused on enzyme activity, but the conclusions about the effects of PEF on enzyme inactivation from different research groups are inconsistent or even contradictory (Yang, Li, & Zhang, 2004a, 2004b; Zhong et al., 2007). To explain the inconsistent results reported by different research groups and further to unveil the mechanisms of enzyme inactivation by PEF, more research work is still required and more detailed information about enzyme inactivation induced by PEF is necessary. Firstly, the different PEF parameters and

* Corresponding author. Address: School of Food Science and Technology, Southern Jiangnan University (Formerly Southern Yangtze University), No. 170, Huihe Road, Wuxi 214036, China. Tel./fax: +86 510 85919150.
E-mail address: yrj@jiangnan.edu.cn (R. Yang).

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.02.008

environmental factors may result in the inconsistent results on enzyme activity, so the factors affecting the inactivation of enzyme by PEF are needed to be investigated. Secondly, understanding the effect of PEF on the structure of enzymes is necessary to elucidate enzyme inactivation mechanisms. In recent years, some researchers have started to study the effects of PEF on enzyme structure. Yeom, Zhang, and Dunne (1999) found the α-helix of secondary structure of PEF-treated papain was decomposed. Zhong, Hu, Zhao, Chen, and Liao (2005) and Zhong et al. (2007) reported the inactivation of PEF-treated horseradish peroxidase, peroxidase and polyphenol oxidase was related to the loss of α-helix of secondary structure. Yang et al. (2004a) reported the inactivation of pepsin by PEF was correlated with the alteration of the secondary structure (β-sheet dominant structure) of pepsin. These studies have looked at the effect of PEF on the structure (in particular secondary structure) of enzymes but this is an area which has not been fully elucidated and the effect on tertiary structure has not really been investigated.

In this study, lysozyme was selected as a model enzyme to investigate the effect of PEF on the activity and structure of enzyme. Lysozyme is of interest for use in food systems since it is a naturally occurring enzyme with antimicrobial activity. It is an important enzyme that has been shown to be effective as a preservative of cheeses, cow's milk, beer (Makki & Durance, 1996), salads and wines (Davidson, 2001). Lysozyme is also the main protein ingredient, performing as inherent preservative in many food products such as egg white and milk. PEF would be applied to these products to reduce microbial load, so it is necessary to understand the effects of PEF on its activity and structure. Moreover, lysozyme was chosen because of its availability and our detailed knowledge of its molecular properties including sequence and structure. Lysozyme is a relatively small globular, monomeric protein, containing structure elements (three stretches of α-helix, an antiparallel pleated sheet, and a sequence folded in irregular way) commonly found in proteins (Torreggiania et al., 2005). In addition, lysozyme is very stable against heat due to its compactly folded molecule (Makki & Durance, 1996); it is easy to differentiate any thermal effect from nonthermal effects of PEF on enzyme.

The objectives of this study were to (1) investigate the factors affecting the inactivation of lysozyme by PEF, (2) determine the effect of PEF on the structure of lysozyme, including the tertiary and secondary structure, and (3) further clarify the connection between enzyme activity reduction and change in secondary structure of lysozyme induced by PEF.

2. Materials and methods

2.1. Materials

Hen egg white lysozyme (3× crystallized, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was used. Freeze-dried *Micrococcus lysodeikticus* powder (M-3770) was obtained from Sigma (St Louis, MO, USA). To dissolve lysozyme and *M. lysodeikticus* powder, sodium phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m at 25 °C determined with a conductivity meter (Hydac, Cambridge Scientific Instruments, Cambridge, Maryland) as the basic medium was used in this study. For the investigations of the effects of electrical conductivity on lysozyme inactivation by PEF, sodium phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.12 S/m adjusted by adding sodium chloride (extremely small amount) was also used. The lysozyme concentration of 2.5 μM served for all experiments, which increased to 5.4 μM to investigate the effects of enzyme concentration on lysozyme inactivation by PEF. All other chemicals used were of reagent grade.

2.2. Bench scale PEF processing system

A bench scale continuous system (OSU-4L, The Ohio State University, Columbus, Ohio, USA) with square-wave pulses was used in this study. After going through each pair of chambers the treated sample was cooled by passing through a coiled tube with a 2.3 mm inner diameter, which was submerged in heat exchange bath (Fisher Scientific Inc., Pittsburgh, PA, USA) with cold water (5 °C). The pre and post-PEF exposure temperatures ($T_{inlet} - T_{outlet}$) at the inlet and outlet of the treatment chamber were measured using a K-type thermocouple (OMEGA, Stamford, CT). The highest temperature achieved in all of the tests was lower than 60 °C (40–57 °C). In this study the flow rate, pulse repetition rate and pulse width were set 0.5 mL/s, 1000 Hz and 2 μs, respectively.

2.3. Lysozyme activity assay

Lysozyme activity was determined by the turbidimetric assay method as described by Maňas, Muňoz, Sanz, and Condón (2006), measuring the decrease in absorbance at 450 nm of a *M. lysodeikticus* suspension versus time with a UV–vis spectrophotometer (UV1201, Beijing Ruili Instrument Co., Beijing, China). A fresh suspension of *M. lysodeikticus* [18 mg solid in 100 mL of phosphate buffer (10 mM, pH 6.2)] was used as substrate. The lysozyme concentrations of the samples served for experiments were 2.5 and 5.4 μM. For each sample, 2.3 mL of substrate were placed in a cuvette held at 25 °C. At time zero, 0.3 mL of lysozyme sample, adequately diluted according to its expected activity, was added to give a total reaction volume of 2.6 mL and shaken quickly. Absorbance measurements were collected at 0.5 s intervals, plots of the decrease of absorbance versus time were drawn and the activity of each sample was calculated (ΔAbs_{450}/min). One enzyme unit is equal to a decrease in turbidity of 0.001/min at 450 nm, so the specific activity of per milliliter of lysozyme sample was calculated as follows:

$$\text{Units(U)/ml} = \frac{\Delta \text{Abs}_{450}/\text{min} \times 1000}{0.3}.$$

2.4. Heat treatment

To determine the effect of heat produced by PEF treatment on the inactivation of lysozyme, lysozyme samples (5 mL, 2.5 μM) were placed in a 25 × 200 mm glass test tube (Pyrex Borosilicate No. 48, Fisher Scientific) and heated in a water bath at 60 °C for 30 s. A type K thermocouple was inserted in the center of the tube. Once the temperature of lysozyme solution reached the desired temperature, the heating time was counted. Then the tube was removed immediately and cooled in 0 °C ice–water bath until tested for activity.

2.5. Relative residual activity

The relative residual activity (RRA) of lysozyme was defined as a percentage of activity of the PEF or heat treated lysozyme solution relative to that of the control. The control was kept in a 0 °C ice–water bath.

2.6. UV absorption spectra and second-derivative spectroscopy

Both zero-order and second-derivative UV spectra of untreated (control) and PEF-treated lysozyme samples (2.5 μM) in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m were obtained with a UV–vis Cary BIO400 (Varian, Palo Alto, CA, USA) at room temperature (25 °C). Phosphate buffer used to dissolve lysozyme was used as blank solution for all of the samples.

2.7. Measurement of intrinsic fluorescence

Intrinsic fluorescence spectra of untreated (control) and PEF-treated lysozyme samples (2.5 μM) in phosphate buffer (10 mM, pH 6.2) were measured at room temperature (25 °C) with a RF-5301PC fluorescence spectrometer (Shimadzu, Kyoto, Japan) at 295 nm (excitation wavelength, slit = 2.5 nm), 300–600 nm (emission wavelength, slit = 2.5 nm) and 10 nm/s of scanning speed. Phosphate buffer used to dissolve lysozyme was used as blank solution for all of the samples.

2.8. Circular dichroism (CD) analysis of lysozyme

CD spectra were scanned at the far-UV range (250–200 nm) with a CD spectropolarimeter (Jasco J-715, Jasco Corp., Tokyo, Japan) in a 0.1 cm quartz CD cuvette (Hellma, Muellheim, Baden, Germany) at 25 °C. The concentration of protein for CD analysis was 2.5 μM. Phosphate buffer used to dissolve lysozyme was used as blank solution for all of the samples. The values of scan rate, response, bandwidth, and sensitivity were 50 nm/min, 0.25 s, 1.0 nm, and 0.1°, respectively. Five scans were averaged to obtain one spectrum. The CD data were expressed in terms of molar ellipticity, $[\theta]$, in deg cm^2/dmol. The molecular ellipticity at 208 nm ($[\theta]_{208\ \text{nm}}$) is a standard measure of helical content of a protein and has been used to estimate the secondary structural change of the protein. The decrease in the intensity of the negative band at 208 nm represents the decrease in the content of α-helix and changes in the secondary structure of lysozyme (Peng, Hidajat, & Uddin, 2004; Zhong et al., 2005). The α-helical content of lysozyme was calculated from the $[\theta]$ value at 208 nm using the equation:

$$\%\text{helix} = \frac{-([\theta]_{208} + 4000)}{29{,}000} \times 100$$

as described by Greenfield and Fasman (1969).

The relative α-helical content (RHC) was calculated as the following equation:

$$\%\text{RHC} = \frac{\text{content of } \alpha\text{−helix after PEF treatment}}{\text{content of } \alpha\text{−helix before PEF treatment}} = \frac{-([\theta]_{208} + 4000)_{\text{After PEF}}}{-([\theta]_{208} + 4000)_{\text{Before PEF}}}.$$

2.9. Statistical analyses

Analysis of variance (ANOVA) was performed using the General Linear Models procedures (GLM) of the Statistical Analysis System (SAS, version 8.0, 2000, Cary, NC, USA). Experiments were triplicated and the means of the three data sets are presented.

3. Results and discussion

3.1. Factors affecting the inactivation of lysozyme by PEF

3.1.1. Effects of electric field strength and treatment time on lysozyme inactivation

Effects of electric field strength and treatment time on the activity of lysozyme (2.5 μM) in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m are shown in Fig. 1A. The results showed that PEF had a significant ($p < 0.05$) effect on the activity of lysozyme, and the activity reduction was a function of the applied electric field strength and treatment time. The RRA of lysozyme steadily decreased as the treatment time increased at a fixed electric field strength, indicating that the longer the PEF treatment time, the greater was the reduction of lysozyme activity. The electric field strength also had significant effects ($p < 0.05$) on the activity of lysozyme. The maximal values of reduction in the activity of lysozyme were 38.1%, 27.7% and 19.2% at 35, 30 and 25 kV/cm for 1200 μs under test conditions, respectively. Previous study (Yang et al., 2004b) showed no significant change in lysozyme activity following 126 μs of PEF treatment (0–38 kV/cm) which

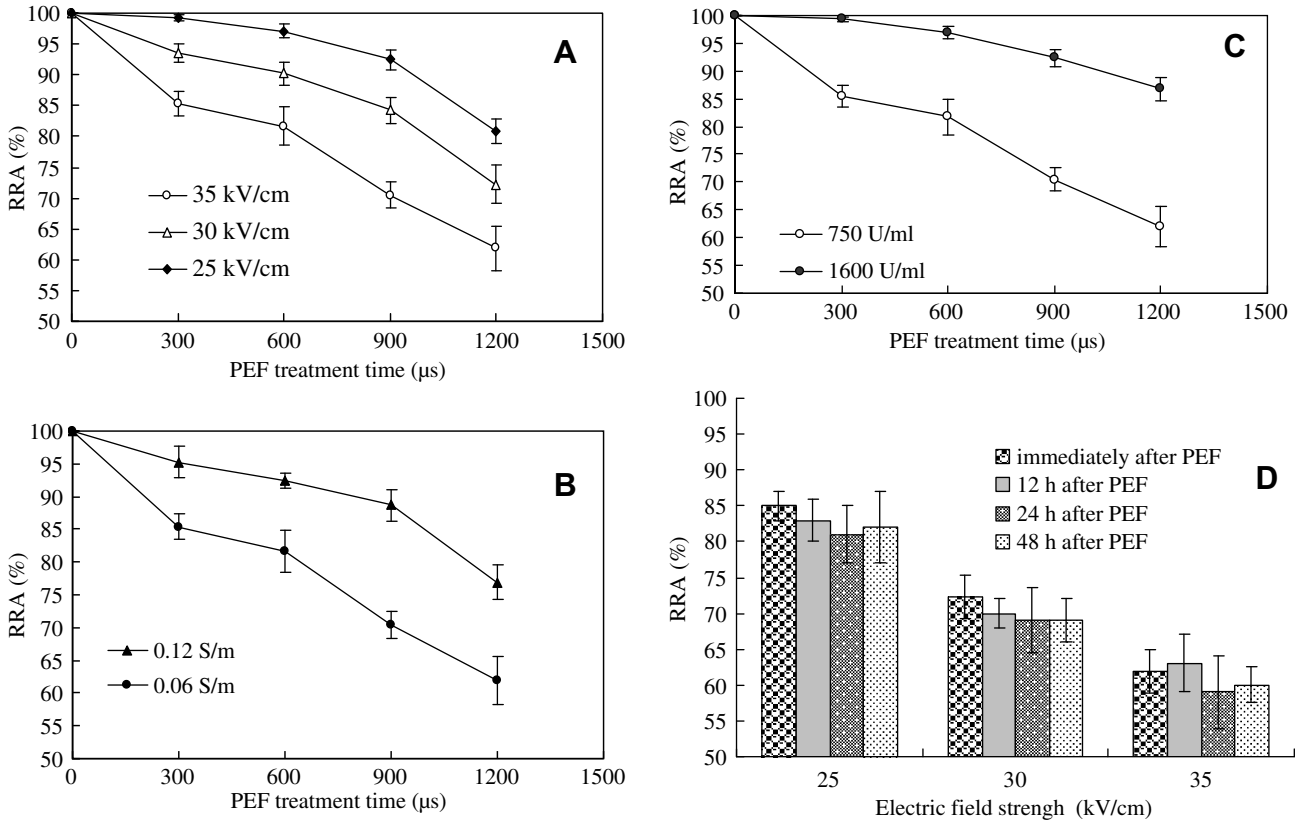

Fig. 1. (A) Effects of electric field strength and treatment time on the inactivation of lysozyme (2.5 μM) in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m. (B) Effects of electrical conductivity on the inactivation of lysozyme (2.5 μM) in phosphate buffer (10 mM, pH 6.2) exposed to PEF treatment at 35 kV/cm for varied treatment time from 300 to1200 μs. (C) Effects of enzyme concentration on the inactivation of lysozyme in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m exposed to PEF treatment at 35 kV/cm for varied treatment time from 300 to1200 μs. (D) Effects of storage time on the PEF-treated lysozyme (2.5 μM) in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m at 4 °C.

was most likely due to insufficient treatment time to inactivate lysozyme. The increase of lysozyme activity under a certain range of voltage in PEF treatment reported previously (Ho, Mittal, & Cross, 1997) was not observed.

In addition, PEF is a nonthermal technology, however, an increase in temperature (e.g., 30–40 °C) can be produced by PEF treatment depending on the sample composition and the processing condition, if the enzyme is heatlabile, it is difficult to differentiate any thermal effect from nonthermal effects of PEF on enzyme (Yeom et al., 1999). The highest temperature achieved in all of the tests shown in Fig. 1 was lower than 60 °C. Lysozyme is very stable against heat due to its compactly folded molecule (Makki & Durance, 1996). To differentiate the effect of heat produced by PEF treatment on the inactivation of lysozyme, further experiments were conducted to keep lysozyme samples in a water bath at 60 °C for 30 s. The result showed that no change in activity occurred at 60 °C. This indicates that the contribution of heat produced during PEF treatment to the inactivation of lysozyme is not significant, and the efficiency of PEF on inactivating enzymes is exclusively related to the PEF treatment.

3.1.2. Effects of electrical conductivity on lysozyme inactivation

Sodium phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m was used as the basic medium for all experiments in this study. The lysozyme solutions with an electrical conductivity of 0.12 S/m were obtained by adding sodium chloride (extremely small amount). Fig. 1B illustrates the effects of electrical conductivity on the inactivation of lysozyme (2.5 μM) in phosphate buffer (10 mM, pH 6.2) exposed to PEF treatment at 35 kV/cm for varied treatment time from 300 to 1200 μs. The results showed that electrical conductivity was an important factor that sufficiently ($p < 0.05$) influenced the lysozyme inactivation by PEF. The activity reduction of lysozyme decreased with the increase of electrical conductivity at the same electric field strength for the same treatment time. For example the minimum RRA of lysozyme sample with an electrical conductivity of 0.06 S/m was 61.9%; however, that of lysozyme sample with an electrical conductivity of 0.12 S/m was 71.9% at 35 kV/cm for 1200 μs under test conditions.

It is known that the electrical conductivity of the medium is one of factors that influence microbial inactivation by PEF, and the higher reduction in survivabilities

observed in liquids with lower conductivities (Dutreux et al., 2000; Korolczuk et al., 2006; Vega-Mercado et al., 1997). One of the potential reasons is that when a constant input voltage is delivered between two electrodes, the effective electric field strength achieved is influenced by the conductivity (Álvarez, Pagán, Condón, & Raso, 2003). Korolczuk et al. (2006) obtained the similar conclusions using a continuous PEF equipment with square waveform pulses. The explain from an electrical point of view was given that, the overall resistance depends on product conductivity, and the higher the electrical conductivity, the lower the resistance. A lower resistance could lead to a negative reflection of the pulse shape and a lower efficiency of the treatment because the system is under loaded. On the other hand, if the resistance is higher, the decreasing phase of the pulse can be approximated by an exponential, leading to an apparent larger pulse width and thus increased treatment efficiency. Sepulveda, Guerrero, and Barbosa-Cánovas (2006) further proved that the variations in electrical conductivity of the treated media could lead to unnoticed modification of the applied treatment because of the high interdependence of the electric field intensity, treatment time, and electrical conductivity of the treated media, and this is responsible for the enhancement of PEF treatment efficiency in a lower conductivity. It was also observed in our research that when the product conductivity is lower, the shape of the pulse is almost square, but the shape becomes irregular with the increase of conductivity. These may be the explanation for the effects of electrical conductivity on rate and extent of lysozyme inactivation by PEF. Further research is needed to explain the mechanisms.

To investigate the effect of electrical conductivity on lysozyme inactivation without PEF treatment, further experiments were conducted to compare activities of lysozyme samples in 0.06 and 0.12 S/m. No difference was observed before and after heat treatment (60 °C, 30 s), indicating that the change in electrical conductivity without PEF treatment in this study could not cause lysozyme inactivation.

3.1.3. Effects of enzyme concentration on lysozyme inactivation by PEF

Lysozyme samples with an enzyme activity of approximate 750 and 1600 U/mL were subjected to PEF treatment, respectively. Fig. 1C illustrates the effects of enzyme concentration on the inactivation of lysozyme in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m exposed to PEF treatment at 35 kV/cm for varied treatment time from 300 to 1200 μs. The results showed that lysozyme concentration sufficiently ($p < 0.05$) influenced the lysozyme inactivation by PEF. The activity reduction of lysozyme decreased when the concentration of lysozyme increased at the same electric field strength for the same treatment time. It indicates that lysozyme in high concentration is more stable against PEF treatment. The potential reason is that the increase of the protein concentration would stabilize the tertiary structure of proteins; in other word at high concentration the presence of the proteins can work as macro ions to stabilize effectively the native conformation (Hirai, Arai, & Iwase, 2000).

3.1.4. Effects of storage time on the activity of PEF-treated lysozyme

The RRA of PEF-treated lysozyme (2.5 μM) was measured after storage for 12, 24 and 48 h at 4 °C. Fig. 1D illustrates the effects of storage time on the activity of PEF-treated lysozyme in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m at 4 °C. No significant ($p > 0.05$) change was observed in RRA of lysozyme samples exposed to 1200 μs of PEF treatment at 25, 30 and 35 kV/cm after storage for 12, 24 and 48 h at 4 °C. It indicates that the PEF conditions examined induced irreversible inactivation of this enzyme. This result was in agreement with the previous reports (Zhong et al., 2005, 2007), but it was not consistent 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). This difference is probably due to different enzymes.

3.2. Effects of PEF treatment on the structure of lysozyme

3.2.1. Effects of PEF treatment on the tertiary structure of lysozyme

Generally, enzymes are globular proteins whose catalytic activity relies on the native configuration of their active site and the conformation of surrounding proteins. The amino acid group present in enzyme proteins creates highly asymmetric spatial distributions of charge that lead to strongly polar and charged regions in the molecular structure of proteins (Giner et al., 2002; Laberge, 1998). The electric fields may cause protein unfolding and denaturation, because of the charge separation (Barsotti & Cheftel, 1999; Giner et al., 2002). Changes in enzyme activity may occur due to the effect of PEF on the 3-dimensional molecular structure of enzymes. The change in structure of lysozyme protein could be reflected by the movement of side chains. Some of the previously buried side chains in the interior of the compact globular region of the native lysozyme protein could be exposed to the surface of molecular, a more polar environment, after PEF treatment. In a contrary manner, some side chains located in the surface could be surrounded with other chains induced by PEF, forming a more hydrophobic environment. Hen egg white lysozyme contains three tyrosine and six tryptophan residues. These aromatic side chains could give rise to some interesting features in the spectra, which can reflect changes in the environment of this side chains. For example, the intrinsic fluorescence of tryptophan residues is particularly sensitive to the polarity of microenvironments along the transition (Viseu, Carvalho, & Costa, 2004). Thereby

tyrosine and tryptophan residues are the most likely candidates to allow one to monitor the change of local tertiary structure. In this study, the conformation changes in lysozyme were evaluated by measuring the UV spectra and intrinsic fluorescence of untreated and PEF-treated lysozyme.

Fig. 2 shows the zero-order (A) and second-derivative (B) UV spectra of untreated and PEF-treated lysozyme (2.5 μM) at 35 kV/cm for 1200 μs. As shown in Fig. 2A, the zero-order UV absorbance spectrum shifted toward lower wavelength after PEF treatment, such as the shift of absorbance peak near 210 nm of control to 204 nm, which indicates the conformational change (Demchenko, 1986). Because of the poor separation of the zero-order absorption bands, direct inspection of protein UV spectra is usually not informative about details of protein tertiary structure. However, spectral resolution is enhanced after numerical derivatization, because sharp bands greatly predominate over broad components (Clérico, Peisajovich, Ceolín, Ghiringhelli, & Ermácora, 2000; Padros, Morros, Manosa, & Dunach, 1982). This technique has been successfully applied to the conformational study of proteins (Granero, Garnero, & Longhi, 2002; Lange & Balny, 2002; Musante et al., 2006). In order to obtain more detailed information on the conformational change of lysozyme induced by PEF, the second-derivative spectroscopy is investigated in Fig. 2B.

Second-derivative spectroscopy has proved to be an effective analytical tool because of its ability to resolve overlapping bands in the normal spectrum into the individual contributions of aromatic side chains, which has been employed for detecting conformational changes involving the microenvironments of aromatic amino acids (Demchenko, 1986; Lange & Balny, 2002; Lopes, Delvivo, & Silvestre, 2005; Ragone, Colonna, Balestrieri, Servillo, & Irace, 1984). Ragone has determined the effects of solvent polarity on the second-derivative spectra of model compounds. For both tyrosine (Tyr) and tryptophan (Trp), as the solvent polarity goes from non-polar (as when the residue is buried inside a protein) to polar (exposed exposed to solvent), the peak and trough positions shift to the blue region (shorter wavelengths). There are also changes in amplitude, which are best described by calculating the ratio ($r = a/b$) of the two peak to trough values (Ragone et al., 1984). As shown in Fig. 2B, the second-derivative spectrum of lysozyme before PEF treatment showed two positive peaks (287 and 298 nm) and two negative troughs (283 and 291 nm). Compared with the peaks of control, all second-derivative peaks of lysozyme after PEF treatment were shifted to lower wavelengths in varying degrees, highlighting the polar environment of the aromatic residues. As shown in Fig. 2B, in the 270–285 nm range, signals from Phe, Tyr and Trp overlap (Clérico et al., 2000; Padros et al., 1982), the large blue shift from 277 to 273 nm was observed induced by PEF. Both Trp and Tyr contribute to the peak at 287 nm (Clérico et al., 2000; Padros et al., 1982), which moved 3 nm to the blue region upon control. The peak at 298 nm can be safely assigned to Trp alone (Clérico et al., 2000; Padros et al., 1982), which showed a blue shift of 1.5 nm after PEF treatment. The above UV absorption results, all second-derivative peaks of lysozyme were shifted to lower wavelengths after PEF treatment was a reflection of unfolding of the tertiary structure induced by PEF.

Peaks around 277 and 285 nm, were assigned to the cooperative contributions of the Trp, Tyr and/or Phe residues. The individual spectroscopic contribution of the Trp or Tyr residues was not established, which was necessary

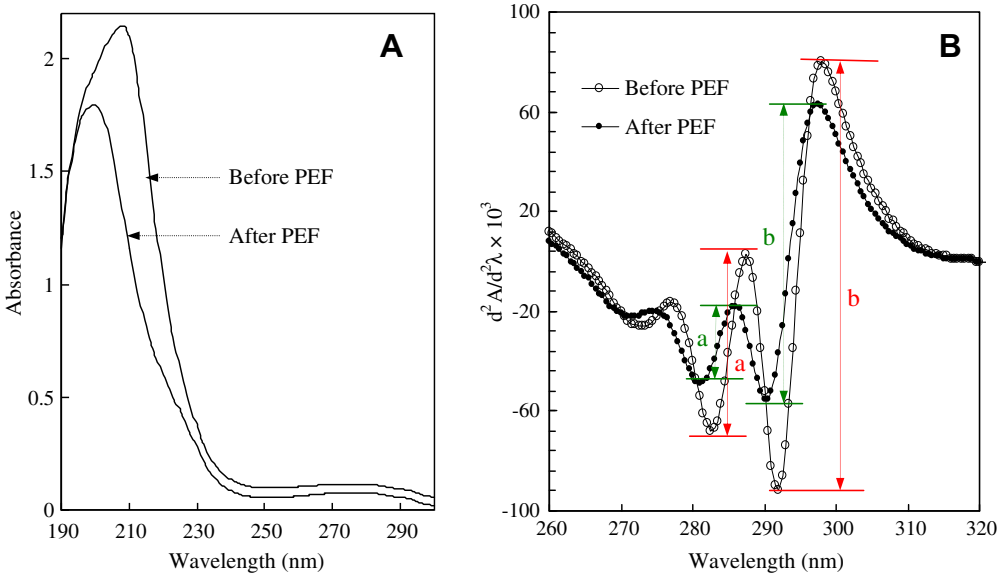

Fig. 2. The zero-order (A) and second-derivative (B) UV spectra of control and PEF-treated lysozyme at 35 kV/cm for 1200 μs. For calculating the ratio ($r = a/b$) of the two peak to trough values, 'a' and 'b' before and after PEF treatment were marked.

for the detailed information of the change in tertiary structure induced by PEF. According to Ragone et al. (1984), dielectric microenvironment surrounding Tyr residues could be calculated from the ratio ($r = a/b$) of the two peak to trough values between differences in second-derivative absorbance peaks. In this study as shown in Fig. 2B, 'a' and 'b' denote arithmetic sum of $d^2A/d\lambda^2$ at 283 and 287 nm, 291 and 298 nm for lysozyme before PEF treatment, respectively. After PEF treatment because of the shift of peaks, 'a' and 'b' denote arithmetic sum of $d^2A/d\lambda^2$ at 280 and 284 nm, 290 and 296.5 nm, respectively. For tyrosine, the value of 'γ' decreases as solvent polarity decreases, while it is almost independent of solvent polarity for tryptophan (Ragone et al., 1984). In this study, the value of 'γ' decreased from 0.41 to 0.26 induced by PEF treatment at 35 kV/cm for 1200 μs. This indicates the change in three-dimensional positions and the movement of Tyr residues of lysozyme to hydrophobic region during the unfolding of the tertiary structure induced by PEF. As is known, there are three tyrosine residues, two of them in the helical domain and one in the beta domain, among the three tyrosine residues, one tyrosine residue is buried in the hydrophobic region in native lysozyme (Zakin & Herschbach, 1986). However, more tyrosine residues were buried inside the protein induced by PEF treatment. Tyr frequently plays a key role in proteins through hydrogen bonding of the hydroxyl group (McHale, 1982). Since Tyr-53 is hydrogen bonded with the amino group of Asp-66 (Blake, Mair, North, Phillips, & Sarma, 1967) and is adjacent to the catalytic residue Asp-52, the changes in the Tyr environment can be associated both with the modification of the environment of the neighbouring Trp residues (Trp-62 and -63).

To further discriminate the spectroscopic contributions of the Trp residues, the conformation changes in lysozyme were evaluated by measuring the intrinsic fluorescence intensity of protein before and after PEF treatment. Fluorescence spectroscopy is a useful technique to follow tertiary structure transitions in proteins because the intrinsic fluorescence of tryptophanyl residues is particularly sensitive to the polarity of microenvironments along the transition (Viseu et al., 2004). In this study, to get rid of possible contributions from Tyr residues, the illumination with 295 nm light exclusively excites tryptophan residues and therefore this spectrum is ascribed to tryptophan (Parisia et al., 2003). As shown in Fig. 3, the fluorescence spectrum of native lysozyme (control) excited at 295 nm had a broad band with a maximum at 335 nm in 10 mM phosphate buffer (pH 6.2). However, the increase of the emission intensity and the red shifts (from 335 to 342 nm) of the maximum were observed after PEF treatment. The similar results of PEF-treated horseradish peroxidase were obtained by Zhong et al. (2005) upon excitement at 234 nm. The results were indicative of the conformational changes in the lysozyme molecule, presumably resulting from the unfolding of the native structure and some of the previously buried Trp residues could be exposed to a more polar environment induced by PEF. It was in agreement with the second-derivative spectra results. As is known, there are six tryptophan residues in lysozyme molecule, and three of them are buried in the hydrophobic region in native lysozyme (Cowgill, 1967; Yokota et al., 2000). However, more tryptophan residues previously buried inside the protein could be exposed to a more polar environment induced by PEF treatment.

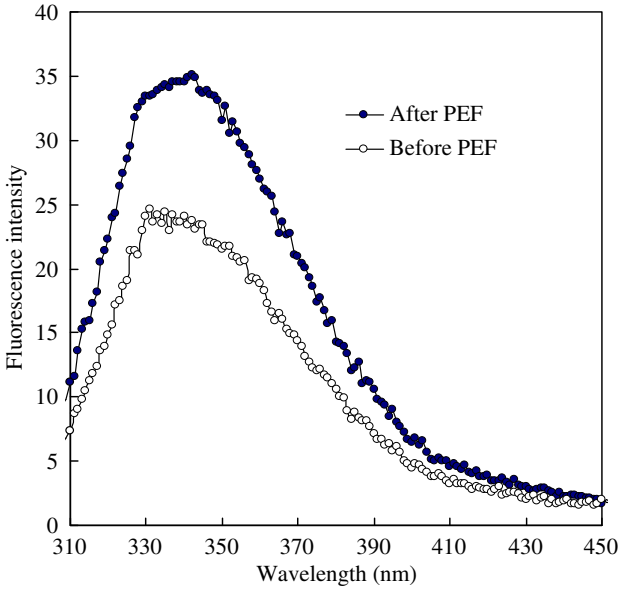

Fig. 3. The intrinsic fluorescence spectroscopy of control and PEF-treated lysozyme at 35 kV/cm for 1200 μs.

From the above results of UV spectra and intrinsic fluorescence, a schematic diagram (Fig. 4) may be useful to illustrate the potential structural changes taking place in the enzyme induced by PEF. As shown in Fig. 4A, Trp-62 and Trp-63 are arranged along one side of the active site, and Trp-108 is in the active cavity on the opposite side of Trp-62 and Trp-63. Trp-28 and Trp-111 are in the hydrophobic region, and Trp-123 is located apart from the others (Cowgill, 1967). Among the six tyrosine residues, three of them are buried in the hydrophobic region in native lysozyme (Cowgill, 1967; Yokota et al., 2000). Two (Tyr-20, Tyr-23) of Tyr residue in the helical domain and one (Tyr-53) in the beta domain, among the three tyrosine residues, one tyrosine residue is buried in the hydrophobic region in native lysozyme (Zakin & Herschbach, 1986). After PEF treatment (Fig. 4B), the unfolding of tertiary structure was occurred, however, the movements of aromatic amino acid side chains were inconsistent, such as more tyrosine residues were surrounded with other chains induced by PEF, and on the contrary more tryptophan residues became exposed after PEF treatment.

3.2.2. Effect of PEF treatment on the secondary structure of lysozyme

Circular dichroism (CD) spectroscopy is the most frequently used technique for evaluation of protein conforma-

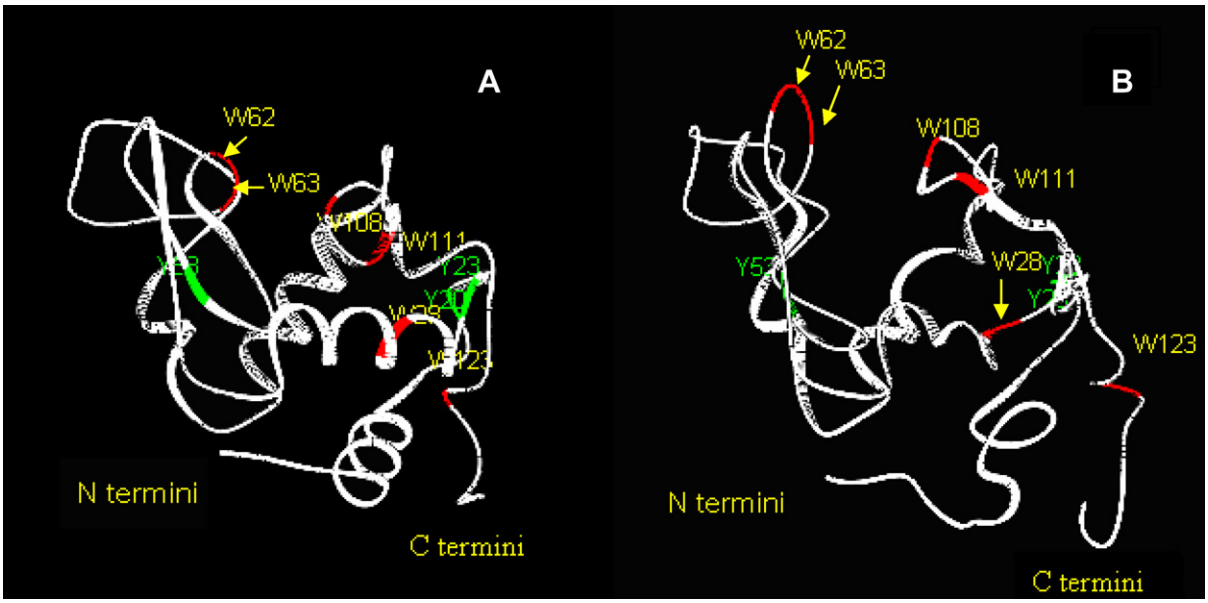

Fig. 4. A schematic diagram of 3-dimensional molecular structure of lysozyme before (A) and after (B) PEF treatment to illustrate the potential structural changes taking place in the enzyme induced by PEF. Solution structure of hen egg white lysozyme (PDB code: 1GXX). The locations of tryptophan and tyrosine residues are indicated in "red" and "green" colors, respectively. The picture was generated by DS Visualizer 1.7 (Accelrys) (W28, 62, 63, 108, 111 and 123 represent Trp-28, 62, 63, 108, 111 and 123 residues, respectively; Y20, 23 and 53 represent Tyr-20, 23 and 53 residues, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tion in solution. The method has been proven to be sufficiently simple and reliable for rapid determination of protein structure or monitoring conformational changes (Townend, Kumosinski, Timasheff, Fasman, & Davidson, 1966). The far-UV CD spectrum is directly related to the protein secondary structure, due to asymmetrical packing of intrinsically achiral (planar) peptide groups (Townend et al., 1966).

Lysozyme, a small monomeric globular protein, its structure is compact with several helices surrounding a small beta sheet region. The active site is formed at the interface between α and β-domains (Yokota et al., 2000), so the secondary structure is important for the activity of lysozyme. Fig. 5 shows the far-UV CD spectra of control and PEF-treated lysozyme at 25, 30, 35 kV/cm for 1200 μs. The spectra of control displays negative CD bands in a wavelength range shorter than 240 nm, which are characterized mainly by two negative bands at 208 and 222 nm. It is identical to that of lysozyme in its native state obtained from Venyaminov and Vassilenko (1994). Fig. 5 illustrates that the intensity of the negative peak of 208 nm decreased after PEF treatment, indicating a loss of α-helical structure induced by PEF. As shown in Fig. 5 the negative peak of 222 nm disappeared with the emergence of a weak positive band between 220 and 230 nm after PEF treatment at 35 kV/cm for 1200 μs, a characteristic of structure of unordered protein (Venyaminov & Yang, 1996), suggests that PEF treatment caused the loss of ordered structure and an increase of the disorder structure content in lysozyme protein. From the above results, it is apparent that the effects of PEF treatment on lysozyme caused a conformational change of the protein with the loss stability of some

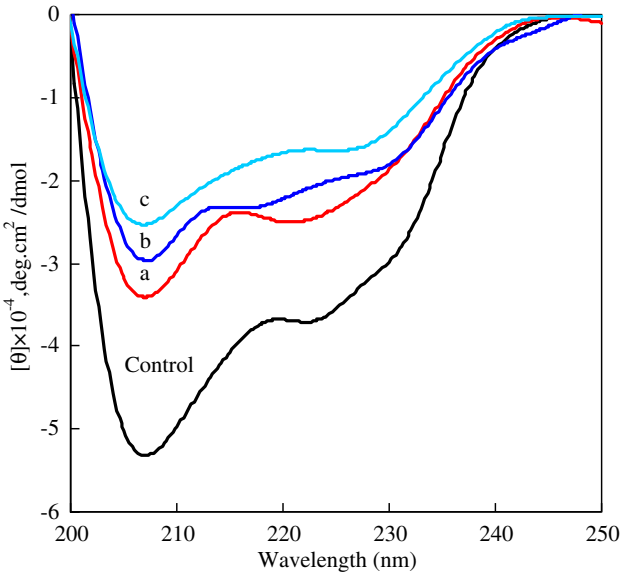

Fig. 5. Far-UV CD spectra of control and PEF-treated lysozyme at (a) 25, (b) 30, (c) 35 kV/cm for 1200 μs.

amount of α-helical structure, as reflected in the schematic diagram (Fig. 4).

In order to further clarify the connection between enzyme activity reduction and secondary structure change induced by PEF, α-helix relative content (RHC) of lysozyme before and after PEF treatment was calculated. The data in Table 1 showed that with the increment of applied electric field strength from 25 to 35 kV/cm for 1200 μs, the RHC and RRA decreased gradually. Table 1 further illus-

Table 1
RRA and RHC of PEF-treated lysozyme

PEF treatment[a] (kV/cm)	RRA[b] (%)	RHC[c] (%)
25	80.8	60.8
30	72.3	52.2
35	61.9	43.0

[a] PEF treatment at each applied electric field strength for 1200 μs.
[b] The relative residual activity of lysozyme.
[c] The relative α-helix content of lysozyme.

trates the relationship between RHC and RRA of PEF-treated lysozyme. These data showed that when the RRA values were 80.8%, 72.3% and 61.9%, the corresponding RHC values were 60.8%, 52.2% and 43.0%, respectively. The RRA decreased with the decrease of RHC of lysozyme in the PEF treatment. This means the change in activity of lysozyme was closely related to the loss of secondary structure in PEF treatment.

In this study, the PEF treatment time never exceeded to 1200 μs, and the highest temperature achieved in all of the tests was lower than 60 °C (40–57 °C). Lysozyme is very stable against heat due to its compactly folded molecule (Makki & Durance, 1996). Many previous studies have proved that lysozyme protein denaturation temperature in the pH range 3.5–7.0 is higher than 70 °C (Back, Oakenfull, & Smith, 1979; Delben & Crescenzi, 1969; Fujita & Noda, 1992; Petersen, Jonson, Fojan, Wimmer, & Pedersen, 2004). The UV, intrinsic fluorescence and CD spectrum of heat treated (60 °C, 30 s) lysozyme samples were also determined, no change of each spectrum with that of control was found, indicating that the effect of heat produced during PEF treatment on these conformational changes could be ruled out.

4. Conclusion

The inactivation of lysozyme in phosphate buffer induced by PEF was a function of applied electric field strength, treatment time, electrical conductivity, and enzyme concentration. The effect of PEF on the 3-dimensional molecular structure of lysozyme may be responsible for modifications in enzyme activity. The unfolding of tertiary structure was induced by PEF, however, the changes in microenvironment of aromatic amino acid side chains were inconsistent, indicating the different movements of side chains of lysozyme protein induced by PEF, such as more tyrosine residues were surrounded with other chains induced by PEF, and on the contrary more tryptophan residues became exposed after PEF treatment. The inactivation of lysozyme by PEF was also correlated to change in secondary structure of lysozyme. The activity decrease of lysozyme was closely related to the loss of α-helix of secondary structure.

Acknowledgements

We sincerely thank Prof. Howard Q. Zhang of USDA for the help of PEF system. This research work was supported by the Project BK2006017 of Jiangsu Provincial National Natural Science Foundation. The authors also gratefully acknowledge the financial support provided by National Key Project of Scientific and Technical Supporting Programs Funded by Ministry of Science and Technology of China during the 11th Five-year Plan (Nos. 2006BAD05A17 and 2006BAD05A02).

References

Álvarez, I., Pagán, R., Condón, S., & Raso, J. (2003). The influence of process parameters for the inactivation of *Listeria monocytogenes* by pulsed electric fields. *International Journal of Food Microbiology, 87*, 87–95.

Ayhan, Z., Yeom, H. W., Zhang, Q. H., & Min, D. B. (2001). Flavor, color, and vitamin C retention of pulsed electric field processed orange juice in different packaging materials. *Journal of Agricultural and Food Chemistry, 49*, 669–674.

Back, J. F., Oakenfull, D., & Smith, M. B. (1979). Increased thermal stability of proteins in the presence of sugars and polyols. *Biochemistry, 18*, 5191–5196.

Barbosa-Canovas, G. V., Pothakamury, U. R., Palou, E., & Swanson, B. G. (1998). *Nonthermal preservation of foods*. New York: Marcel Dekker.

Barsotti, L., & Cheftel, J. C. (1999). Food processing by pulsed electric fields. II. Biological aspects. *Food Review International, 15*, 181–213.

Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1967). On the conformation of the hen egg-white lysozyme molecule. *Proceedings of the Royal Society of London. Series B, 167*, 365–377.

Clérico, E. M., Peisajovich, S. G., Ceolín, M., Ghiringhelli, P. D., & Ermácora, M. R. (2000). Engineering a compact non-native state of intestinal fatty acid-binding protein. *Biochimica et Biophysica Acta, 1476*, 203–218.

Cortés, C., Esteve, M. J., Frígola, A., & Torregrosa, F. (2005). Quality characteristics of horchata (a Spanish vegetable beverage) treated with pulsed electric fields during shelf-life. *Food Chemistry, 91*, 319–325.

Cowgill, R. W. (1967). Fluorescence and protein structure. *Biochimica et Biophysica Acta, 140*, 37–44.

Davidson, P. M. (2001). Chemical preservatives and natural antimicrobial compounds. In M. P. Doyle (Ed.), *Food microbiology* (pp. 601–602). Washington, DC: ASM Press.

Delben, F., & Crescenzi, V. (1969). Thermal denaturation of lysozyme. A differential scanning calorimetry investigation. *Biochimica et Biophysica Acta, 194*, 615–618.

Demchenko, A. P. (1986). *Ultraviolet spectroscopy of protein*. Germany: Springer-Verlag.

Dutreux, N., Notermans, S., Wijtzes, T., Góngora-Nieto, M. M., Barbosa-Cánovasb, G. V., & Swanson, B. G. (2000). Pulsed electric fields inactivation of attached and free-living *Escherichia coli* and *Listeria innocua* under several conditions. *International Journal of Food Microbiology, 54*, 91–98.

Elez-Martínez, P., & Martín-Belloso, O. (2007). Effects of high intensity pulsed electric field processing conditions on vitamin C and antioxidant capacity of orange juice and gazpacho, a cold vegetable soup. *Food Chemistry, 102*(1), 201–209.

Fujita, Y., & Noda, Y. (1992). Effect of alkylation with different sized substituents on thermal stability of lysozyme. *International Journal of Peptide and Protein Research, 40*, 103–109.

Giner, J., Ortega, M., Mesegué, M., Gimeno, V., Barbosa-Cánovas, G. V., & Martín, O. (2002). Inactivation of peach polyphenoloxidase by exposure to pulsed electric fields. *Journal of Food Science, 67*(4), 1467–1472.

Granero, G., Garnero, C., & Longhi, M. (2002). Second derivative spectrophotometric determination of trimethoprime and sulfamethoxazole in the presence of hydroxypropyl-b-cyclodextrin (HP-b-CD). *Journal of Pharmaceutical and Biomedical Analysis, 29*, 51–59.

Greenfield, N., & Fasman, G. D. (1969). Computed circular dichroism spectra for the evaluation of protein conformation. *Biochemistry, 8*, 4108–4116.

Hirai, M., Arai, S., & Iwase, H. (2000). Concentration dependence of thermal structural transition of hen egg-white lysozyme under constant heating rate studied by time-resolved SAXS. *Thermochimica Acta, 344*, 95–102.

Ho, S. Y., Mittal, G. S., & Cross, J. D. (1997). Effect of high field electric pulses on the activity of selected enzymes. *Journal of Food Engineering, 31*, 69–84.

Jeyamkondan, S., Jayas, D. S., & Holley, R. A. (1999). Pulsed electric field processing of foods: A review. *Journal of Food Protection, 62*, 1088–1096.

Knorr, D. (1992). Novel approaches in food-processing technology: New technologies for preserving foods and modifying function. *Current Opinion in Biotechnology, 10*, 485–491.

Korolczuk, J., Keag, J. R. M., Fernandez, J. C., Baron, F., Grosset, N., & Jeantet, R. (2006). Effect of pulsed electric field processing parameters on Salmonella enteritidis inactivation. *Journal of Food Engineering, 75*, 11–20.

Laberge, M. (1998). Intrinsic protein electric fields: basic noncovalent interactions and relationship to protein-induced Stark effects. *Biochimica et Biophysica Acta, 138*, 305–350.

Lange, R., & Balny, C. (2002). UV–visible derivative spectroscopy under high pressure. *Biochimica et Biophysica Acta, 1595*, 80–93.

Lopes, D. C. F., Delvivo, F. M., & Silvestre, M. P. C. (2005). Use of activated carbon for removing phenylalanine from reconstituted skim milk powder hydrolysates. *LWT – Food Science and Technology, 38*, 447–453.

Makki, F., & Durance, T. D. (1996). Thermal inactivation of lysozyme as influenced by pH, sucrose and sodium chloride and inactivation and preservative effect in beer. *Food Research International, 29*, 635–645.

Maňas, P., Muňoz, B., Sanz, D., & Condón, S. (2006). Inactivation of lysozyme by ultrasonic waves under pressure at different temperatures. *Enzyme and Microbial Technology, 39*, 1177–1182.

McHale, J. L. (1982). Fermi resonance of tyrosine and related compounds. Analysis of the Raman doublet. *Journal of Raman Spectroscopy, 13*, 21–24.

Mertens, B., & Knorr, D. (1992). Developments of nonthermal processes for food preservation. *Food Technology, 46*, 125–133.

Musante, L., Bruschi, M., Candiano, G., Petretto, A., Dimasi, N., Boccio, P. D., et al. (2006). Characterization of oxidation end product of plasma albumin 'in vivo'. *Biochemical and Biophysical Research Communications, 349*, 668–673.

Padros, E., Morros, A., Manosa, J., & Dunach, M. A. (1982). The state of tyrosine and phenylalanine residues in proteins analyzed by fourth-derivative spectrophotometry. *Histone H1 and ribonuclease A. European Journal of Biochemistry, 127*, 117–122.

Parisia, M., Mazzinia, A., Sorbia, R., Ramonic, R., Grollic, S., & Favillaa, R. (2003). Unfolding and refolding of porcine odorant binding protein in guanidinium hydrochloride: Equilibrium studies at neutral pH. *Biochimica et Biophysica Acta, 1652*, 115–125.

Peng, Z. G., Hidajat, K., & Uddin, M. S. (2004). Adsorption and desorption of lysozyme on nano-sized magnetic particles and its conformational changes. *Colloids and Surfaces B: Biointerfaces, 35*, 169–174.

Petersen, S. B., Jonson, V., Fojan, P., Wimmer, R., & Pedersen, S. (2004). Sorbitol prevents the self-aggregation of unfolded lysozyme leading to an up to 13 °C stabilisation of the folded form. *Journal of Biotechnology, 114*, 269–278.

Qin, B., Zhang, Q., Barbosa-Canovas, G., Swanson, B., & Pedrow, P. (1995). Pulsed electric field treatment chamber design for liquid food pasteurization using a finite element method. *Transactions of the ASAE, 38*(2), 557–565.

Ragone, R., Colonna, G., Balestrieri, C., Servillo, L., & Irace, G. (1984). Determination of tyrosine exposure in proteins by second derivative spectroscopy. *Biochemistry, 23*, 1871–1875.

Sepulveda, D. R., Guerrero, J. A., & Barbosa-Cánovas, G. V. (2006). Influence of electric current density on the bactericidal effectiveness of pulsed electric field treatments. *Journal of Food Engineering, 76*, 656–663.

Torreggiania, A., Tambaa, M., Mancob, I., Rosaria, M., Faraone-Mennellab, C., Ferreria, C., et al. (2005). Radiation damage of lysozyme in a biomimetic model: Some insights by Raman spectroscopy. *Journal of Molecular Structure, 744–747*, 767–773.

Townend, R., Kumosinski, T. F., Timasheff, S. N., Fasman, G. D., & Davidson, B. (1966). The circular dichroism of the beta structure of poly-L-lysine. *Biochemical and Biophysical Research Communications, 23*, 63–69.

Vega-Mercado, H., Martín-Belloso, O., Qin, B., Chang, F. J., Góngora-Nieto, M. M., Barbosa-Cánovas, G. V., et al. (1997). Non-thermal food preservation: Pulsed electric fields. *Trends in Food Science & Technology, 8*, 151–157.

Venyaminov, S. Y., & Vassilenko, K. S. (1994). Determination of protein tertiary structure class from circular dichroism spectra. *Analytical Biochemistry, 222*, 176–184.

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

Viseu, M. I., Carvalho, T. I., & Costa, S. M. B. (2004). Conformational transitions in b-lactoglobulin induced by cationic amphiphiles: equilibrium studies. *Biophysical Journal, 86*, 2392–2402.

Yang, R. J., Li, S. Q., & Zhang, Q. H. (2004a). Effects of pulsed electric fields on the activity and structure of pepsin. *Journal of Agricultural and Food Chemistry, 52*, 7400–7406.

Yang, R. J., Li, S. Q., & Zhang, Q. H. (2004b). Effects of pulsed electric fields on the activity of enzymes in aqueous solution. *Journal of Food Science, 69*, 241–248.

Yeom, H. W., Zhang, Q. H., & Dunne, C. P. (1999). Inactivation of papain by pulsed electric fields in a continuous system. *Food Chemistry, 67*, 53–59.

Yokota, A., Izutani, K., Takai, M., Kubo, Y., Noda, Y., Koumoto, Y., et al. (2000). The transition state in the folding–unfolding reaction of four species of three-disulfide variant of hen lysozyme: The role of each disulfide bridge. *Journal of Molecular Structure, 295*, 1275–1288.

Zakin, M. R., & Herschbach, D. R. (1986). Vibrational frequency shifts induced by molecular compression of pyridine in solution. *Journal of Chemical Physics, 85*, 2376–2383.

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

Zhong, K., Wu, J., Wang, Z., Chen, F., Liao, X., Hu, X., & Zhang, Z. (2007). Inactivation kinetics and secondary structural change of PEF-treated POD and PPO. *Food Chemistry, 100*, 115–123.