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Investigation of the Mechanisms of Pulsed Electric Fields on Inactivation of Enzyme: Lysozyme

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Lysozyme was selected as a model enzyme to investigate the effects of pulsed electric fields (PEF) on its activity and structure. The irreversible inactivation of lysozyme in sodium phosphate buffer (10 mM, pH 6.2) induced by PEF at 35 kV/cm followed a first-order model when the treatment time was longer than 300 μs. Unfolding of lysozyme structure was induced by PEF, accompanied by the cleavage of disulfide bonds and self-association aggregation when the applied PEF dosage was higher than a critical level. The inactivation of lysozyme by PEF was correlated to the loss of α-helix in secondary structure. The relative residual activity of PEF-treated lysozyme was in close agreement with the relative molar ellipticity at 208 nm. Both PEF- and heat-induced inactivations of lysozyme were correlated to the alteration of the secondary structure of lysozyme, but the effects of PEF and heat treatment on secondary structure were inconsistent.

KEYWORDS: Pulsed electric fields (PEF); lysozyme; inactivation; aggregation; unfolding; secondary structure

INTRODUCTION

Pulsed electric fields (PEF) is a nonthermal food-processing technology to inactivate microorganisms and enzymes (*1*–*5*). It is a potential complement to or replacement of thermal pasteurization and has undergone substantial developments near commercial application (*6*, *7*). 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 (*8*, *9*). However, compared with the extensive studies on the inactivation of microorganisms by PEF, there are limited reports about the effects of PEF on enzymes. The mechanisms involved in inactivation of enzymes by PEF are not fully understood at present. Understanding the effects of PEF on the enzymes is necessary for the further development and commercialization of PEF processing.

It was proved in our previous paper that the sensitivity of enzymes to PEF did not relate to their thermal stability, size, or whether they have quaternary structure, suggesting that the inactivation mechanism of enzymes by PEF may be different from that of thermal inactivation (*10*). In recent years, some researchers have studied the effects of PEF on enzyme structure and have tried to establish some relationships between enzyme activity reduction and structure change induced by PEF. Some of them also further investigated the effects of PEF and heat on secondary structure of enzymes. Yeom et al. (*11*) found that inactivation of PEF-treated papain was related to the loss of α-helix structure. In our previous paper, we (*5*) reported the inactivation of pepsin by PEF followed a first-order model. Circular dichroism analysis showed that both PEF- and heat-induced inactivations of pepsin were correlated with the alteration of the secondary structure (β-sheet dominant structure) of pepsin. Zhong et al. (*12*) reported that the α-helix relative contents of horseradish peroxidase 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. The reduction of α-helix relative content caused by heating 100 °C for 5 min was greater than that by PEF treatment. Zhong et al. (*13*) observed that the inactivations of PEF-treated peroxidase and polyphenol oxidase were related to the conformational change of secondary structure. However, the information is not enough to elucidate enzyme inactivation mechanisms, and the comparison between PEF- and heat-induced changes in the structure of enzyme seems to be vague. More detailed information about enzyme structure before and after PEF treatment is necessary.

In this study, lysozyme was selected as a model enzyme to investigate the mechanism of enzyme inactivation by PEF. Lysozyme is extensively used in the pharmaceutical, biochemical, and food fields. Its physicochemical properties and structure are very clear. Lysozyme is a relatively small globular, monomeric protein (molecular weight 14300) containing 129 amino acids, of which 3 are tyrosine, 6 tryptophan, and 2 methionine, and it contains four pairs of cysteine residues, forming four intramolecular disulfide bonds. The simplicity of the lysozyme structure makes it easy to understand changes in activity and structure. Lysozyme contains structure elements commonly found in proteins. The single polypeptide chain of lysozyme is mainly of three structural components. The first

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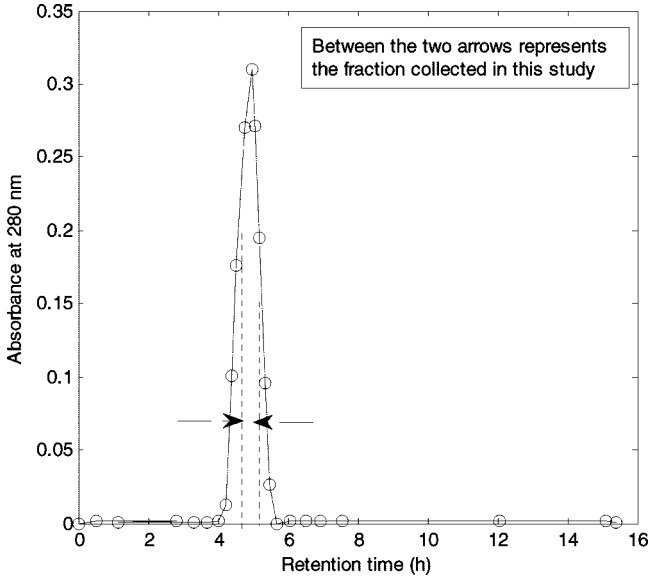


Figure 1. Elution profile of lysozyme from Sephadex G-50. The lysozyme powder (3× crystallized) was dissolved in phosphate buffer (10 mM, pH 6.2), loaded on a previously equilibrated Sephadex G-50 gel filtration column (1.5 cm × 100 cm). Elution was carried out with the same buffer at a flow rate of 25 mL/h. Protein fractions were monitored at 280 nm.

component consists of three stretches of α-helix, the second is an antiparallel pleated sheet, and a third is a sequence folded in an irregular way (*14*, *15*). In addition, PEF is a nonthermal technology; however, an increase in temperature (e.g., up to 30–40 °C) can be produced by PEF treatment depending on the sample composition and the processing condition. If the enzyme is heat-labile, it is difficult to distinguish any thermal effect from nonthermal effects of PEF on enzyme (*11*). Lysozyme is remarkably highly stable against heat due to its compactly folded molecule (*16*). The objectives of this study were to (i) investigate effects of PEF on the activity and structure of lysozyme, (ii) correlate the reduction in activity and the change in secondary structure of lysozyme during PEF treatment, and (iii) compare effects on secondary structure of lysozyme induced by PEF with those by heat, when approximately equivalent activity reductions of lysozyme samples were obtained by PEF and heat treatment, respectively.

MATERIALS AND METHODS

Materials. Hen egg white lysozyme (3× crystallized, Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) was used with further purification performed by Sephadex G-50 gel filtration chromatography. Freeze-dried *Micrococcus lysodeikticus* powder (M-3770) was obtained from Sigma (St. Louis, MO). All other chemicals used were of reagent grade.

Further Purification Procedure and Preparation of Lysozyme Samples. Hen egg white lysozyme (100 mg) diluted into 5 mL of cooled (4–6 °C) sodium phosphate buffer (10 mM, pH 6.2) was loaded on a previously equilibrated Sephadex G-50 gel filtration column (Pharmacia, Uppsala, Sweden) (1.5 cm × 100 cm) with the same phosphate buffer. The column was run using the same phosphate buffer at a flow rate of 25 mL/h. Protein elution was monitored at 280 nm on a UV spectrophotometer (365A, Shanghai Science Optical Instrument Co., Ltd., Shanghai, China). The central fraction of the absorbance peak was collected as shown in **Figure 1**. The collected lysozyme solution certified a single band by sodium dodecyl sulfate–polyacrylamide gel electrophoresis was diluted with the same phosphate buffer (10 mM, pH 6.2) to obtain lysozyme samples with expected enzyme activity prior to use. In this study lysozyme samples with an enzyme activity of approximate 750 units/mL and an electrical conductivity of 0.06 S/m determined with a conductivity meter at 25 °C (Hydac, Cambridge Scientific Instruments, Cambridge, MD) were used for all experiments.

Protein Concentration Assay. Protein concentration was spectrophotometrically determined on the basis of extinction coefficient at 280 nm of 2.635 cm^2/mg for lysozyme solutions (*17*).

Lysozyme Activity Assay. Lysozyme activity was determined by the turbidimetric assay method as described by Maňas et al. (*18*), measuring the decrease in absorbance at 450 nm of a *M. lysodeikticus* suspension versus time in a UV–vis spectrophotometer (UV1201, Beijing Ruili Instrument Co., Beijing, China). A fresh suspension of *M. lysodeikticus* [18 mg of solid in 100 mL of sodium phosphate buffer (10 mM, pH 6.2)] was used as substrate. For each sample, 2.3 mL of substrate was 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). The specific activity per milliliter of lysozyme sample was calculated as follows:

$$\text{units(U)/mL} = \frac{\Delta Abs_{450}/\text{min} \times 1000}{0.3} \quad (1)$$

Bench-Scale PEF Processing System. A bench-scale continuous system (OSU-4L, The Ohio State University, Columbus, OH) was used. The schematic diagram of the system is shown in **Figure 2A**. A model 9310 trigger generator (Quantum Composer Inc., Bozeman, MT) was used to control frequency and pulse width and delay time between opposite polarities. Signals of voltage, current, frequency, and waveform were monitored by a two-channel 1 GS/s (60 MHz bandwidth) digital real-time oscilloscope (model TDS 210, Tektronix Inc., Wilsonville, OR). **Figure 2B** shows a typical set of voltage and current waveforms used in this study. Six cofield flow tubular chambers with a 2.92 mm electrode gap and a 2.3 mm inner diameter were grouped in three pairs, and each pair was connected with stainless steel tubing with a 2.3 mm inner diameter. After going through each pair of chambers, the treated sample was cooled by passage through a coiled tube with a 2.3 mm inner diameter, which was submerged in a heat exchange bath (Fisher Scientific Inc., Pittsburgh, PA) with cold water (5 °C). The pre- and post-PEF exposure temperatures (T_{inlet} and T_{outlet}) at the inlet and outlet of the treatment chamber were measured using a K-type thermocouple (OMEGA, Stamford, CT). The maximum average elevation temperature ($DT = T_{outlet} - T_{inlet}$) during the PEF treatments was 55 °C in this study. The apparatus was thoroughly cleaned with 70% ethyl alcohol and rinsed with sterile distilled water after each experimental run. A micro gear pump (model 020-000-010, Micropump, Inc., Vancouver, WA) maintained a continuous flow of sample. PEF treatment time (t) was calculated with the number of pulses received in the chambers (Np), which is obtained from residence time in a chamber (Tr) as

$$\text{Tr} = V/F \quad (2)$$

where V is the volume of a chamber (mL) and F is the flow rate (mL/s)

$$\text{Np} = \text{Tr} \times f \quad (3)$$

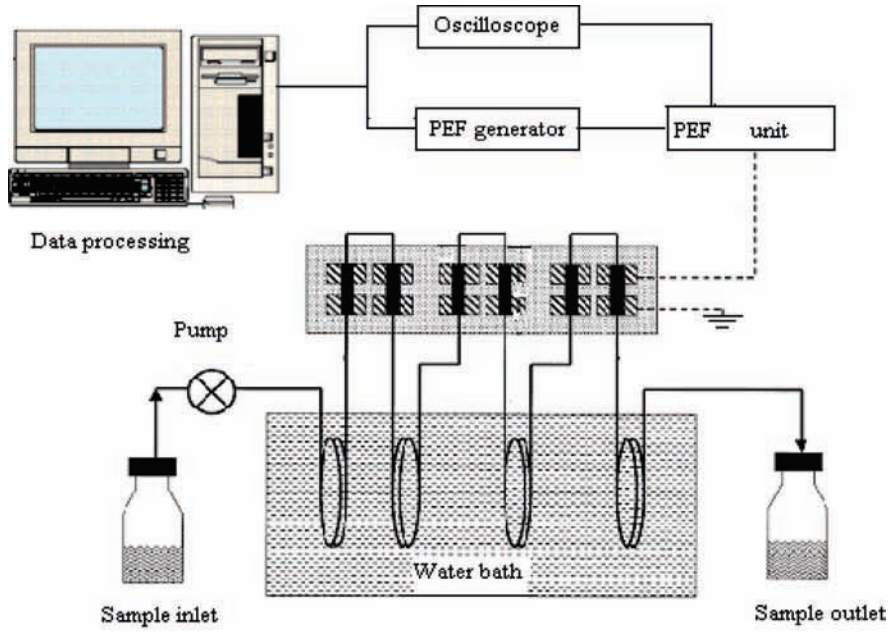
where f is the pulse repetition rate (pulses per second, pps)

$$t = \text{Np} \times \text{Nc} \times \text{Wp} \quad (4)$$

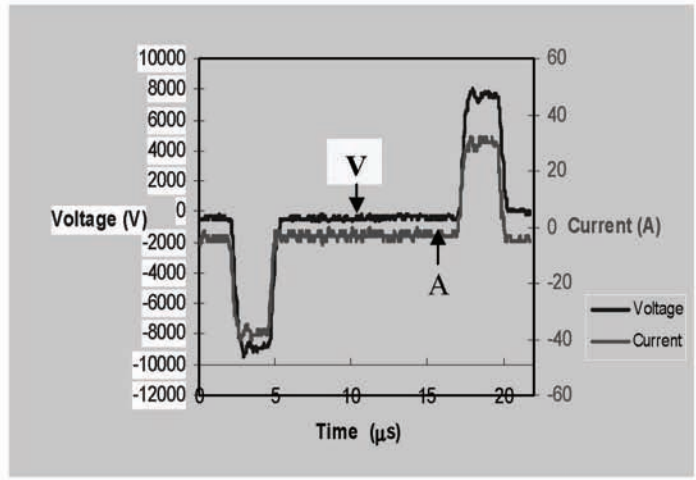
where Nc is the number of treatment chambers and Wp is the pulse width (μs).

In this study the flow rate, pulse repetition rate, and pulse width were set at 0.5 mL/s, 1000 pps, and 2 μs, respectively.

Heat Treatment. To determine the effect of heat produced by PEF treatment on the inactivation of lysozyme, lysozyme samples (5 mL) were placed in a tube and heated in a water bath at 60 °C for 30 s. A type K thermocouple was inserted in the center of the tube. The heating time was counted after the temperature of lysozyme solution reached the desired temperature. Then the tube was removed immediately and cooled in a 0 °C ice–water bath until tested for activity. To investigate



(A)



(B)

Figure 2. Diagram of (**A**) PEF bench-scale processing unit and (**B**) bipolar square wave pulse pair.

the different effects of PEF and heat on the secondary structural change of lysozyme, lysozyme samples (5 mL) were heated in a water bath at 100 °C for different times as above. It is known that thermal inactivation of lysozyme approached first-order kinetics (*18*), so lysozyme samples with enzyme activity reduction equivalent to that of samples treated by PEF can be obtained by heat treatment.

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. Prior to activity assay, all samples were kept in a 0 °C ice–water bath.

First-Order Inactivation Kinetics of Lysozyme by PEF. The experimental data were fit to the first-order inactivation models as described in our previous paper (5):

$$\ln(\mathrm{RRA}) = -k_N \times t \quad (5)$$

The inactivation rate constant (k_N) was obtained from the slope of the regression of ln(RRA) as compared to PEF treatment time (t), where t is the PEF treatment time (μs) and k_N is the first-order kinetic constant.

Relative Protein Concentration. The relative protein concentration (RPC) of lysozyme was defined as a percentage of the concentration of soluble protein of the PEF-treated lysozyme solution to that of the control. RPC was calculated as

$$\mathrm{RPC} = A_{280}/A_{C280} \quad (6)$$

where A_{280} is the absorbance at 280 nm of the PEF-treated lysozyme solution filtered with a 0.45 μm syringe filter (Gelman Science, Ann Arbor, MI) and A_{C280} is the absorbance at 280 nm of the control filtered with the same model syringe filter. A_{280} and A_{C280} were measured by a UV–vis spectrophotometer (UV1201, Beijing Ruili Instrument Co.) at room temperature.

Analysis of Exposed Sulfhydryl Groups. Exposed sulfhydryl groups (SH) of lysozyme samples before and after PEF treatment were measured according to the method of Beveridge et al. (*19*). Ellman's reagent was prepared by dissolving 40 mg of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma Chemical Co.) in 10 mL of methanol. One milliliter of sample was added to 5 mL of Tris-Gly buffer (86 mM Tris and 90 mM Gly, pH 8.0). Subsequently, 40 μL of Ellman's reagent was added for color reaction. The mixture was shaken up and incubated

at room temperature (25 °C) for 10 min, and then absorbance was measured at 412 nm on a UV–vis spectrophotometer (UV1100, Beijing Ruili Instrument Co.). Reagent blank and sample blank were initially prepared to correct the color from reagents and protein solution. SH content was calculated according to the equation

$$\mu\text{mol of SH/g} = (73.53A_{412})D/c \quad (7)$$

where A_{412} is the absorbance at 412 nm, C is the sample concentration in mg/mL, and D is the dilution factor.

Measurement of Surface Hydrophobicity (S_0). The method described by Hayakawa and Nakai (*20*) was employed to determine S_0 of PEF-treated and nontreated (control) lysozyme samples (2.5 μM), using 1-anilinonaphthalene-8-sulfonate (ANS) (Sigma Chemical Co.) as a hydrophobic probe.

Measurement of Intrinsic Fluorescence. Intrinsic fluorescence spectra of PEF-treated and nontreated (control) lysozyme samples were measured at room temperature (25 °C) in phosphate buffer (10 mM, pH 6.2) with an 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.

Circular Dichroism (CD) Analysis. 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 molar ellipticities of lysozyme samples were calculated as described in our previous paper (*5*). All lysozyme samples treated by PEF or heat were measured immediately, and the activity of each sample was determined before and after CD analysis to ensure no change in activity during measurement of CD.

Relative Molar Ellipticity at 208 nm. The relative molar ellipticity at 208 nm ($R[\theta]_{208\ nm}$) of lysozyme was defined as a percentage of the molar ellipticity at 208 nm ($[\theta]_{208\ nm}$) of the PEF- or heat-treated lysozyme relative to that of the control.

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). Experiments were carried out in triplicate, and the means of the three data sets are presented.

RESULTS AND DISCUSSION

Effect of PEF on the Activity of Lysozyme. The effects of PEF treatment on the activity of lysozyme in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m at 35 kV/cm are shown in **Figure 3**. The results in **Figure 3A** show that there was no reduction in the activity of lysozyme induced by PEF before 150 μs at 35 kV/cm. Prolonging the treatment time, PEF had a significant ($p < 0.05$) effect on the activity of lysozyme. The reductions of the activity of lysozyme were 8, 20, 29.5, and 38.1% at 35 kV/cm for 300, 600, 900, and 1200 μs under test conditions, respectively. The RRA of lysozyme steadily decreased as the treatment time increased, indicating that the longer the treatment time of PEF treatment, the greater was the reduction of lysozyme residual activity. The increase of lysozyme activity under a certain range of voltage in PEF treatment reported previously (*21*) was not observed. In our previous paper (*10*) no significant change in lysozyme activity was observed after PEF from 0 to 38 kV/cm for 126 μs, owing to a lack of enough PEF dosage such as treatment time to inactivate the lysozyme. In addition, no significant ($p > 0.05$) change was observed in RRA of PEF-treated lysozyme samples, after storage for 24 and 48 h at 4 °C. This indicates that

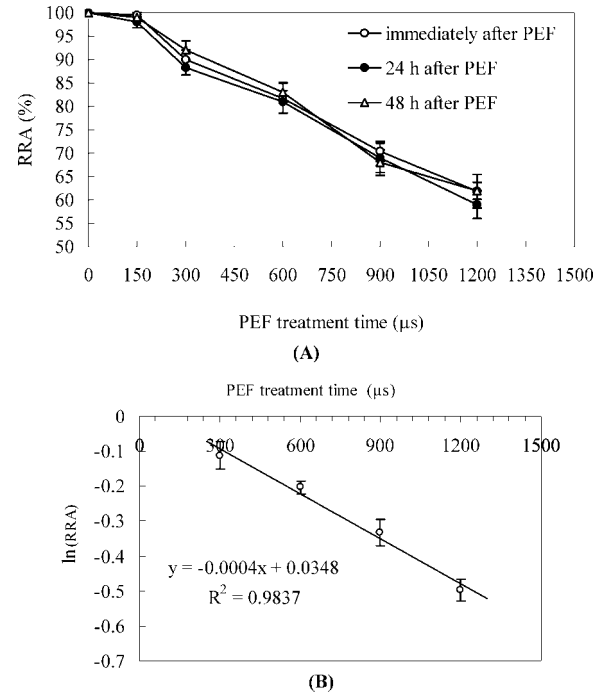


Figure 3. Effect of PEF treatment on the activity of lysozyme in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m: (**A**) inactivation of lysozyme exposed to PEF for varied treatment time from 0 to 1200 μs at 35 kV/cm measured immediately after PEF treatment and after storage for 24 and 48 h at 4 °C; (**B**) inactivation kinetics of PEF-treated lysozyme at applied electric strength of 35 kV/cm.

PEF-induced inactivation of lysozyme was irreversible. This result was in agreement with the previous reports for horseradish peroxidase, peroxidase, and polyphenol oxidase inactivated by PEF (*12*, *13*), 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 (*11*).

As shown in **Figure 3B**, the RRA values as a function of PEF treatment time were greatly fit to the first-order inactivation model in eq 5 when the total treatment time is higher than 300 μs. The correlation coefficient (γ^2) of the first-order inactivation model was 0.9837, which suggests that the first-order inactivation model may be suitable for describing the inactivation of lysozyme by PEF at the applied electric field strength when PEF treatment time is higher than a critical level. The first-order inactivation kinetic constant k_N of lysozyme in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m at 35 kV/cm was 0.0004 (1/μs). The trend line of ln(RRA) versus treatment time (t) suggests that the first-order inactivation kinetics is valid when, at 35 kV/cm, the treatment time is longer than 300 μs, which indicates that to inactivate lysozyme, PEF dosage should be higher than a critical level, which may be defined as critical dosage for the inactivation of lysozyme by PEF.

The highest temperature of lysozyme samples in all of the tests shown in **Figure 3** was lower than 60 °C. Lysozyme is very stable against heat due to its compactly folded molecule (*16*). To explain 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 of activity occurred at 60 °C (data not shown). This indicates that the contribution

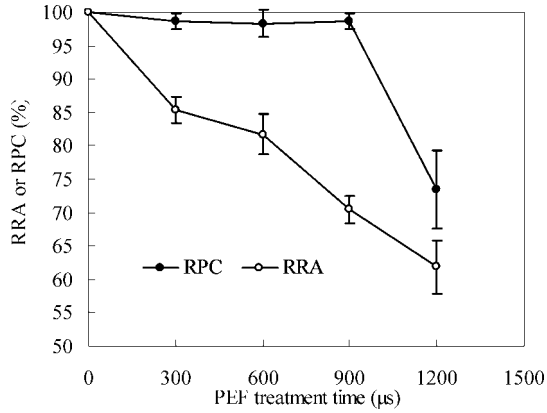


Figure 4. Effects of PEF treatment on the aggregation and activity of lysozyme in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m. The applied electric strength was 35 kV/cm.

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.

Analysis of Aggregation Induced by PEF. Figure 4 shows the effects of PEF treatment on RPC and RRA of lysozyme in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m. The concentration of total protein including the soluble and insoluble was 2.5 μM. As shown in **Figure 4**, the RRA decreased with the increase of treatment time. However, no decrease in RPC was observed before 900 μs of PEF treatment at 35 kV/cm, which means that no aggregation of lysozyme was formed and the inactivation occurred earlier than aggregation did. With prolonged PEF treatment time, RPC started to decrease and decreased to 74.3% when PEF treatment time reached 1200 μs. However, as shown in **Figure 4**, activity decreased much more rapidly than solubility did. This suggests that PEF treatment may cause aggregation of lysozyme protein when the applied PEF dosage is higher than a critical level for the concentration of lysozyme of 2.5 μM in this study, but the inactivation occurred before the protein formed aggregates.

Analysis of Exposed SH Groups Induced by PEF. The lysozyme structure is clearly characterized as a compactly folded molecule, the rigidity of which is stabilized by the four disulfide bonds (6Cys–127Cys, 30Cys–115Cys, 64Cys–80Cys, and 76Cys–94Cys) (*22*). These disulfide bonds are well-known to be stable to denaturing agents and heat treatment, but are easily disrupted with reducing agents, and reduction of these S–S bridges is conducive to a greater molecular flexibility and dramatic increase in exposed hydrophobic regions (*23–25*).

Figure 5 presents the exposed SH groups of lysozyme gradually increased as a function of PEF treatment time. The reactivity of the SH group to Ellman's reagent, which is not accessible to the SH group in the native structure, increased significantly ($p < 0.05$) after PEF treatment. With 300, 600, 900, and 1200 μs of PEF treatment at 35 kV/cm, about 83, 95.7, 159.5, and 216.9 μmol/g of exposed SH were detected, respectively. The results indicate that PEF-induced inactivation of lysozyme was accompanied by the cleavage of the disulfide bonds. Gilquin et al. (*26*) reported that the decrease in enzymatic activity of lysozyme was a function of residual disulfide bonds, as a result of the disruption of the conformational structure forming the active site, due to unfolding of its molecule.

Aggregation occurred when a protein was subjected to pressure and temperature, by creating new disulfide bonds from reactive SH groups (*27–30*). In this study, the application of PEF for a long time (1200 μs) caused obvious aggregation as

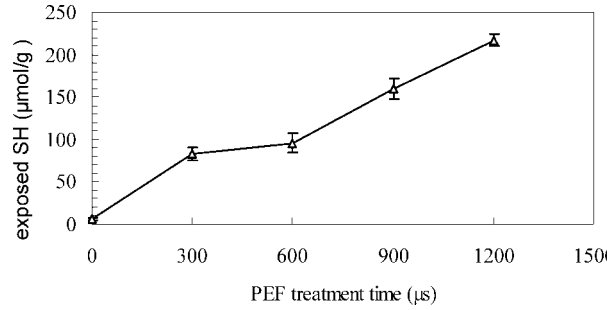


Figure 5. Effects of PEF treatment on exposed SH groups of lysozyme in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m. The applied electric strength was 35 kV/cm.

can be seen from results obtained using RPC (see **Figure 4**). This means that PEF treatments can break the disulfide bonds and cause aggregation when the applied PEF dosage is higher than a critical level.

Analysis of Hydrophobicity (S_0) and Intrinsic Fluorescence. Surface hydrophobicity (S_0) of proteins is one of the structural characteristics used to evaluate the change in protein conformation (*31, 32*). **Figure 6A** shows the surface hydrophobicity (S_0) of lysozyme before and after PEF treatment. The S_0 dramatically increased with increase of PEF treatment time at a constant PEF strength of 35 kV/cm, reaching the maximum when treatment time was 600 μs, but it slightly declined with further increase of PEF treatment time. The S_0 value of native lysozyme (control) was noticeably low in agreement with the measurement by Touch et al. (*33*), because the accessibility of ANS to the hydrophobic residues, most of which are buried in the interior of the compact globular region (*20*), was inhibited. It was, however, remarkable that before 600 μs of PEF treatment, the S_0 value was found to considerably increase, reflective of exposure of hydrophobic amino acid residues buried in the folded structure (*26*). This phenomenon suggested that PEF treatment induced molecular unfolding of lysozyme, destroyed hydrophobic interactions of protein molecules, caused more hydrophobic groups and regions inside the molecules to expose outside, and thus increased S_0 of lysozyme. When PEF treatment time was longer than 600 μs, S_0 decreased, which was the so-called hydrophobic collapse (*34*), indicating that protein self-association aggregation occurred as a consequence of an increased protein–protein interaction via hydrophobic association leading to the formation of dimer, trimer, or oligomer or even precipitation. It was proved by the decrease of lysozyme solubility in **Figure 4**.

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 (*35*). Generally, the fluorescence of protein is due to three intrinsic fluors, tryptophan, tyrosine, and phenylalanine residues. Actually, the intrinsic fluorescence of many proteins is almost contributed by tryptophan alone because the quantum yield of phenylalanine is very low and the fluorescence of tyrosine is almost totally quenched if it is ionized or near an amino group, a carboxyl group, or a tryptophan (*36*). As is known, lysozyme is a small monomeric globular protein formed by 129 tactic amino residues containing 6 tryptophans (*14, 15*). Therefore, tryptophan residues are the most likely candidates to reflect the change of local tertiary structure. In this study, the conformational changes in lysozyme were evaluated by measuring the intrinsic fluorescence intensity of protein before and after PEF treatment. The illumination with 295 nm light exclusively excites

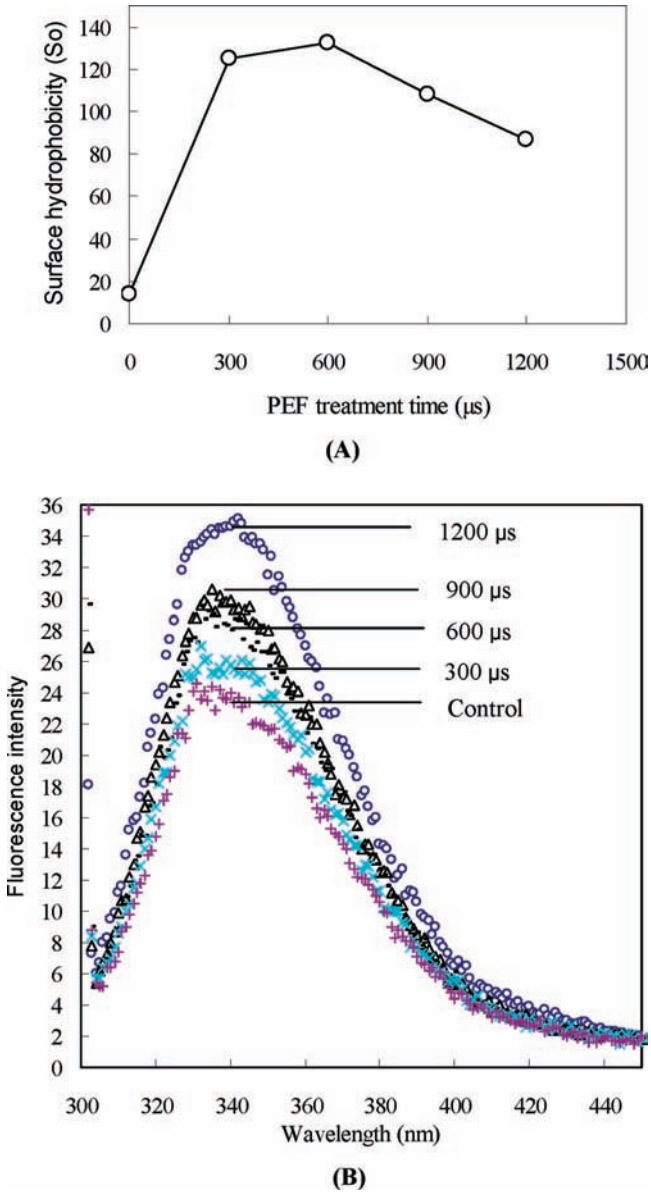


Figure 6. Effects of PEF treatment on the (**A**) surface hydrophobicity (S_0) and (**B**) intrinsic fluorescence of lysozyme in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m. The applied electric strength was 35 kV/cm.

tryptophan residues, and therefore this spectrum is ascribed to tryptophan. The change in local tertiary structure of lysozyme could be obtained by determining intrinsic fluorescence spectroscopy.

As shown in **Figure 6B**, the fluorescence spectrum of native lysozyme (control) excited at 295 nm had a broadband with a maximum at 335 nm in 10 mM phosphate buffer (pH 6.2). However, after PEF treatment, the gradual increases of the emission intensity and the red shifts (from 335 to 342 nm) of the maximum with an increase in PEF treatment time were observed. The intrinsic fluorescence intensity of lysozyme reached the maximum, about 144% of that of the control, under the test conditions, when lysozyme sample was subjected to PEF treatment for 1200 μs at 35 kV/cm. It is a fact that there are six tryptophan residues in the native lysozyme molecule. 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 (*37*). The results obtained from **Figure 6B** were indicative of the conformational changes in the lysozyme molecule, presumably resulting from changes in the microenvironment around lysozyme; that is, some of the previously buried Trp residues, such as Trp-28, -111, and -108, could be exposed to a more polar environment, because of the unfolding of the native structure induced by PEF. The increase in fluorescence intensity may be primarily ascribed to the change in three-dimensional positions of Trp-62 and -108, because Imoto et al. (*38*) showed that 80% of fluorescence of lysozyme was due to Trp-62 and Trp-108. As it is known that Trp-108 is located at the active cavity and Trp-62 is arranged along one side of the active site, in addition, when the enzyme–substrate complex is undergoing catalysis, the –NH of the indole ring of Trp-62 is hydrogen bonded to the oxygen attached to C-6 of tri-*N*-acetylglucosamine, the substrate of lysozyme (*39*). Therefore, the change in locations of Trp-62 and Trp-108 can be reflective of dissociation of the active site and influence the binding of substrate to lysozyme.

The fluorescence of tryptophan at 295 nm is affected by the energy transfer from tyrosine to tryptophan (if the distance between them is <10–18 Å) and also by fluorescence quenching of adjacent groups (*40*). For example, Trp-63 and Trp-23 residues in native lysozyme are near both disulfide linkages, Cys76–Cys94 and Cys6–Cys127, respectively; thereby, their contribution to the fluorescence can be negligible due to the quenching by both near disulfide linkages (*37*). Aother reason for the increase of tryptophan fluorescence might be the movement of tryptophan residues far from the quenchers and/or the movement of tryptophan residues closer to tyrosine residues because of the cleavage of the disulfide bonds and unfolding of the native structure induced by PEF, so that the energy transfer to tryptophan increases, enhancing the quantum yield.

Effect of PEF on the Secondary Structure of Lysozyme. CD is a relatively powerful technique to investigate the secondary structural change of proteins, because they are, in the far-UV region, related to the polypeptide backbone structure of proteins. CD spectra can directly interpret the changes of protein secondary structure (*41*). CD analysis was used to detect the change of the secondary structure of lysozyme after PEF treatment in this study. **Figure 7A** shows the CD spectra of lysozyme samples before and after various times of PEF treatment at 35 kV/cm. The spectrum of control displays negative CD bands in a wavelength range shorter than 240 nm, which is 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 (*42*). These bands are caused by a negative Cotton effect characteristic of helical structure (*43*). The negative bands at 208 and 222 nm are rationalized by the $n\rightarrow\pi^*$ transition in the peptide bond of α-helical structure (*44*). It is noted from **Figure 7A** that with the increase of PEF treatment time from 0 to 1200 μs, the intensity of the negative peak of 208 nm decreased. This indicates a gradual loss of α-helical structure induced by PEF. With the increase of PEF treatment time from 0 to 600 μs, the intensity of the negative peak of 222 nm decreased, and when lysozyme samples were subjected to PEF treatment for 900 and 1200 μs, the negative peak of 222 nm disappeared with the emergence of a weak positive band between 220 and 230 nm, the band attributed to a characteristic structure of unordered protein (*43*). This indicates that PEF treatment caused the loss of ordered structure and an increase of the disordered structure content in lysozyme protein under the tested condition. From the above results, it is apparent that the effect of PEF treatment on lysozyme caused a conformational change of the protein with

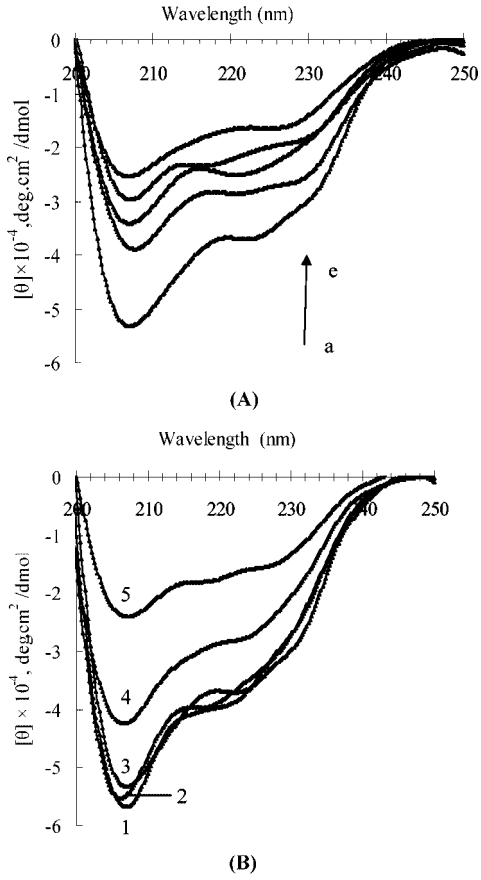


Figure 7. Far-UV CD spectra of lysozyme (**A**) before and after PEF treatment for various times at 35 kV/cm (a, control; b, 300 μs, RRA = 92%; c, 600 μs, RRA = 80%; d, 900 μs, RRA = 70.5%; e, 1200 μs, RRA = 62%) and (**B**) before and after heat treatment (1, control; 2, RRA = 90%; 3, RRA = 78%; 4, RRA = 71%; 5, RRA = 63%) in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m.

the loss of stability of some amount of α-helical structure. Zhong et al. (*12*, *13*) also found the inactivation of PEF-treated horseradish peroxidase, peroxidase, and polyphenol oxidase was correlated to the reduction of α-helix fraction in secondary structure. Yeom et al. (*11*) observed loss of α-helix structure in PEF-treated papain with the significant reduction of papain activity. Neumann and Katchalsky (*44*) reported that electric pulses of about 20 kV/cm induced long-lived helix–coil transition polynucleotide helices. Helix–coil transition of synthetic polypeptides at 30 kV/cm was reported (*45*). Yeom et al. (*11*) explained α-helix structure was susceptible to conformational change by high electric field due to its dipole moment. Each peptide bond carries a dipole moment, and the alignment of peptide bonds in α-helix structure gives rise to a macrodipole of considerable strength (*46*, *47*).

To investigate the different effects of PEF and heat on the secondary structural change of lysozyme, lysozyme samples with RRA approximately equivalent to that of samples treated by PEF were obtained by heat treatment. **Figure 7B** shows the CD spectra of lysozyme before and after heat treatment. With decrease of RRA of lysozyme samples induced by heat, the intensity of the negative peak of 208 nm decreased, which is similar to the results of **Figure 7A**. This indicates that both PEF- and heat-induced inactivations of lysozyme were correlated to the loss of α-helical structure. This is similar to the conclusion in our previous paper (*5*) that the inactivation of pepsin (highly β-sheet dominant protein), no matter whether it is achieved by PEF or heat treatment, is the result of the loss of its secondary

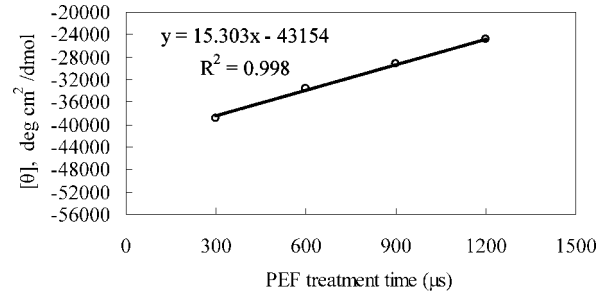


Figure 8. Change in molecular ellipticity at 208 nm ($[\theta]_{208\ nm}$) measured after PEF treatment for various times. The lysozyme samples were in phosphate buffer (10 mM, pH 6.2) with an electrical conductivity of 0.06 S/m. The applied electric strength was 35 kV/cm.

structure. However, compared with PEF treatment, heat treatment slightly changed the secondary structure of lysozyme samples with RRA values of 90 and 78%; the CD characteristics of native lysozyme and heat-treated lysozyme samples with RRA values of 90 and 78% were qualitatively similar (**Figure 7B**). This implies that lysozyme with a small degree of inactivation induced by heat has the same backbone secondary structure as the native protein, but the secondary structure was changed to a large degree when RRA decreased to 71 and 63% induced by heat. The possible reason for the difference with PEF treatment is that the molten-globule state (*48*) may exist in the process of inactivation induced by heat.

An estimate of the α-helix content may be made from the molecular ellipticity at 222 nm (*49*) or 208 nm (*50*). The molecular ellipticity at 208 nm ($[\theta]_{208\ nm}$) 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 (*13*, *37*, *51*). **Figure 8** shows the change in molecular ellipticity at 208 nm ($[\theta]_{208\ nm}$) measured after PEF treatment for various times. It is noted that the baseline of $[\theta]_{208nm}$ markedly slants upward from 300 to1200 μs of PEF treatment, which means that with the increase in PEF treatment time, the loss of α-helical structure gradually occurred. It is noticeable that the $[\theta]_{208\ nm}$ values as a function of PEF treatment time were well fit to the first-order model when the total treatment time is higher than 300 μs. The correlation coefficient (γ^2) of the inactivation model was 0.998, which suggests that the linear model may be suitable for describing the change of $[\theta]_{208\ nm}$ during the process of PEF-induced inactivation of lysozyme at the applied electric field strength when PEF treatment time is higher than a critical level. However, more data were needed to confirm the relationship between PEF treatment and the change of secondary structure.

Figure 9 further illustrates the relationship between relative residual activity (RRA) and relative molar ellipticity at 208 nm ($R[\theta]_{208\ nm}$) of PEF- and heat-treated lysozyme as compared to the control. The results in **Figure 9** show that the RRA decreased with the decrease of $R[\theta]_{208\ nm}$ in both PEF and heat treatment test. It indicates that the inactivation of lysozyme, especially for PEF-induced inactivation, is a function of the degree of change in its secondary structure. With the decrease of RRA, the change of $R[\theta]_{208\ nm}$ presents two stages for both the PEF and the heat treatment tests. In PEF treatment, $R[\theta]_{208\ nm}$ changed quickly when the RRA values decreased from 100 to 92% (at the beginning of inactivation induced by PEF), and then it changed relatively slowly when the RRA values decreased from 92 to 63%. However, $R[\theta]_{208\ nm}$ changed inversely in heat treatment, which decreased slightly when RRA

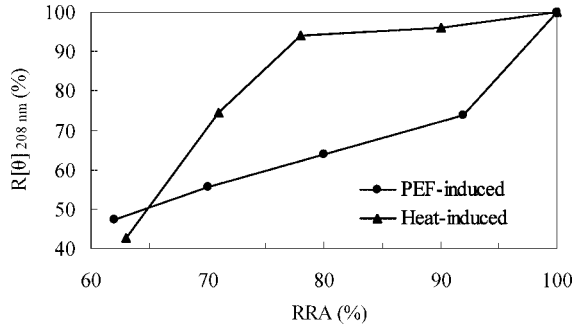


Figure 9. Relationship between relative residual activity (RRA) and relative molar ellipticity at 208 nm ($R[\theta]_{208\ nm}$) of PEF- and heat-treated lysozyme as compared to the control.

decreased from 100 to 78%, and then the change became more rapid when RRA decreased from 78 to 62%. In addition, when the RRA values were similar, such as 92% in PEF treatment and 90.4% in heat treatment, the $R[\theta]_{208\ nm}$ values were remarkably variant, 73.6% in PEF treatment and 95% in heat treatment. This means that although both PEF- and heat-induced inactivations of lysozyme were correlated to the loss of α-helical structure, the effects of PEF and heat treatments on secondary structure are inconsistent. In PEF treatment, secondary structure changed quickly at the beginning of inactivation induced by PEF, and then it changed relatively slowly and linearly with the decrease of RRA (or increase of the PEF treatment time). In heat treatment, lysozyme with a small degree of inactivation induced by heat has the same backbone secondary structure as the native protein, but the secondary structure was changed to a large degree when RRA continued to be decreased by heat. The data in **Figure 9** show that when the RRA values were 92, 80, 70.5, and 62% in PEF treatment, the $R[\theta]_{208\ nm}$ values were 73.6, 63.8, 55.6, and 47.3%, respectively. From these data, another conclusion may be obtained that the activity of lysozyme changed more quickly than its secondary structure in PEF treatment.

Lysozyme, a small monomeric globular protein, has a compact structure with several helices surrounding a small β-sheet region. The active site is formed at the interface between α- and β-domains (*52*). The secondary structure is important for the activity of lysozyme. The change of the content of α-helix during PEF treatment was related to the inactivation of lysozyme.

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