JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Pulsed Electric Fields on the Activity and Structure of Pepsin

RUIJIN YANG,[†,‡] SI-QUAN LI,[†] AND Q. HOWARD ZHANG*,[†]

Department of Food Science and Technology, 2015 Fyffe Road, The Ohio State University, Columbus, Ohio 43210, and School of Food Science and Technology, Southern Yangtze University, 170 Huihe Road, Wuxi, Jiangsu 214036, China

A continuous pulsed electric field (PEF) system integrated with six co-field flow PEF treatment chambers was used to study the inactivation of pepsin. The inactivation of pepsin activity was a function of applied electric field strength, electrical conductivity, and pH. The inactivation of pepsin by PEF followed a first-order model. The first-order inactivation kinetic constant of pepsin was 0.012 (1/μs) in 7.5 mM HCl (pH 2.0) at 34.2 kV/cm. Aggregation of pepsin was observed during PEF treatment; however, the inactivation took place before the formation of aggregates. Circular dichroism analysis showed that inactivation of pepsin by PEF was correlated to the loss of β-sheet structure in a pepsin molecule. The relative residual activity of PEF-treated pepsin was correlated to the relative molar ellipticity at 215 nm. Both PEF- and heat-induced inactivation of pepsin were correlated with the alteration of the secondary structure (β-sheet dominant structure) of pepsin.

KEYWORDS: Pulsed electric fields; pepsin inactivation; CD spectroscopy; secondary structure

1. INTRODUCTION

High voltage pulsed electric fields (PEF) is a nonthermal food processing technology, which has been researched and developed to almost the commercial stage (*1*−*3*). As compared to thermal processing, PEF has a number of advantages including minimal changes in flavor, taste, color, nutrients, and heat labile functional components of foods (*4*−*11*). Therefore, PEF is receiving considerable attention from the food industry.

Understanding the effects of PEF on the enzymes is necessary for the further development and commercialization of PEF processing. Some research was conducted in this aspect, but the conclusions about the effects of PEF on enzymes elucidated by different research groups are inconsistent. Ho and his colleagues (*12*) reported that under the tested conditions (13−87 kV/cm, 0.5 Hz, 2 μs pulse width, 30 pulses), lipase, glucose oxidase, and α-amylase exhibited a significant activity reduction of 70−85%; peroxidase and polyphenol oxidase displayed a moderate 30−40% reduction; alkaline phosphatase only showed a slight 5% reduction; whereas the activity of pepsin was not decreased but significantly increased at 20−45 kV/cm. Van Loey et al. (*13*) reported that lipoxygenase, polyphenol oxidase, pectin-methylesterase, and peroxidase in distilled water are resistant toward PEF, but had significant activity reduction in more complex liquid dairy or fruit and vegetable products. Irreversible reduction of activity was observed in PEF-treated papain after 24 h storage at 4 °C (*14*). The inactivation of PEF treatment papain was related to the loss of α-helix structure. PME activity was inactivated by 90% by PEF treatment at 35 kV/cm for 59 μs in a pilot-plant system (*7*). Significant activity reductions of polyphenol oxidase after bipolar exponential decay PEF treatment were reported, such as a 97% reduction in apple extract at 24.6 kV/cm for 6000 μs, a 72% reduction in pear extract at 22.3 kV/cm for 6000 μs (*15*), and a 70% reduction in peach at 24.30 kV/cm for 5000 μs (*16*). Grahl and Märkl (*17*) reported up to 60% activity reduction of lipase in raw milk at an energy input of 200 kJ/L, but no inactivation of alkaline phosphatase and lactoperoxidase was observed at 21.5 kV/cm and a total energy input of 400 kJ/L. However, 65% activity reduction of alkaline phosphatase in raw milk was observed at 18.8 kV/cm and 70 pulses (*18*). Plasmin activity decreased 90% after PEF treatment of 50 pulses of 2 μs at 30 and 45 kV/cm (*19*). The same research group (*20*) observed an 80% inactivation in tryptic soy broth with yeast extract at 18 kV/cm for 40 μs, but no significant inactivation of protease in casein-Tris buffer. Higher inactivation levels were reached in skim milk than in whole milk, and 81% inactivation was attained in skim milk at 35.5 kV/cm for 866 μs (*21*). In our previous study (*22*) on the effects of PEF on pepsin, polyphenol oxidase, peroxidase, chymotrypsin, and lysozyme using a continuous PEF processor with co-field tubular electrodes, it was observed that PEF treatment had a mild effect on the activity of peroxidase (18.1% reduction at 34.9 kV/cm for 126 μs) and chymotypsin (4.0% reduction at 34.2 kV/cm for 126 μs) and no effect on the activity of lysozyme. However, pepsin and polyphenol oxidase were sensitive to PEF. For pepsin, the activity reduction reached 51.7% and 83.8% at 37.0 kV/cm for 126 μs and 41.8 kV/cm for 126 μs, respectively.

* Author to whom correspondence should be addressed [telephone (614) 688-3644; fax (614) 292-0218; e-mail zhang.138@osu.edu].
† The Ohio State University.
‡ Southern Yangtze University.

10.1021/jf049183p CCC: $27.50
Published on Web 10/28/2004

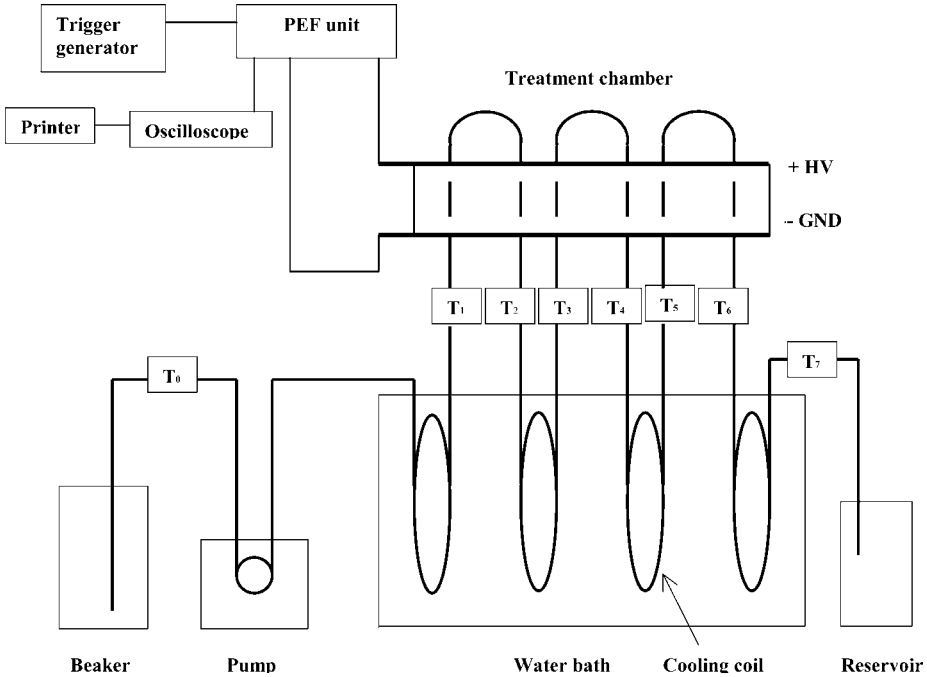

Figure 1. Diagram of the PEF bench-scale processing unit (OSU-4A). T_1–T_6 were measured by thermocouples. T_0 and T_7 were measured by mercury thermometers.

It is necessary to unveil the mechanism of enzyme inactivation by PEF and to explain the inconsistent results reported by different research groups. Most of the research work on enzyme inactivation by PEF is focused on the effect of PEF on the activity. The mechanisms involved in inactivation of enzymes by PEF are not fully understood. To elucidate enzyme inactivation mechanism, information about enzyme structure before and after PEF treatment is necessary.

In this study, pepsin was selected as a model enzyme to investigate the mechanism of enzyme inactivation by PEF. Pepsin is one of the enzymes that is important in meat tenderization (*23*, *24*) and cheese ripening (*25*, *26*) and the production of other milk products (*27*, *28*). Pepsin belongs to the family of the aspartic proteases, which display a high structural homology (*29*). It is a monomeric, two domain, mainly β-protein, with a high percentage of acidic residue (43 out of 327) leading to a very low pI (isoelectric point). The catalytic site consists of two aspartate residues, Asp32 and Asp215, one of which has to be protonated and the other deprotonated, for pepsin to be active (*30*). This occurs at pH 1−4 (*31*). When the pH is higher than 7.0, pepsin is in a denatured conformation; however, it maintains some of its original secondary structure (*32*, *33*). The objectives of the this study were to (a) determine the effect of PEF on the activity and secondary structure of pepsin and (b) correlate the change in activity and the change in secondary structure of pepsin, achieved by PEF treatment.

2. MATERIALS AND METHODS

2.1. Materials. Crystallized, pepsin powder was purchased from Sigma Co. (St. Louis, MO) and was used directly without further purification. All other chemicals were also obtained from Sigma Co.

2.2. Preparation of Pepsin Solutions. Pepsin powder was dissolved in cooled (4−6 °C) media at selected pH and electrical conductivity immediately before PEF treatment or heat treatment. Three media were used in this study, 7.5 mM HCl, 1 mM HCl + NaCl, and water−phosphate−acetate mixture, for pH 2.0, pH 2.9, and pH 3.8, respectively. Electrical conductivity of the media was adjusted using sodium chloride. Water−phosphate−acetate mixture was prepared by adding glacial acetic acid to 20 mM potassium phosphate to pH 3.8. The concentrations of pepsin solutions for activity study were within the values recommended by Sigma for activity assays.

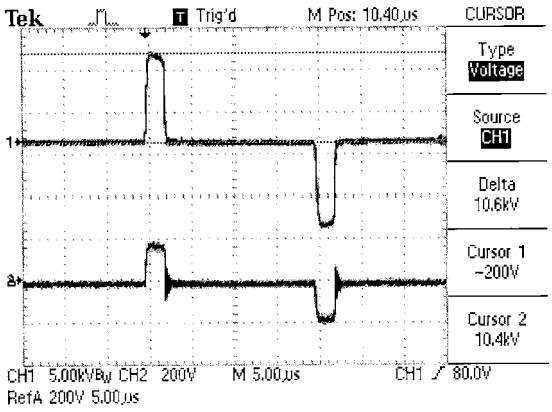

Figure 2. Bipolar square wave pulse pair. Trace 1 is the measured voltage with 5 kV per division. Trace 2 is the measured current with 20 A per division. Time scale is 5 μs per division.

2.3. PEF Treatment. A bench-scale continuous PEF system (OSU-4A, The Ohio State University, Columbus, OH) was used to treat the enzyme solutions. A schematic diagram is shown in **Figure 1**. A model 9310 trigger generator (Quantum Composer Inc., Bozeman, MT) was used to control frequency and pulse duration time and delay time between opposite polarity. 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 2** shows a typical set of voltage and current waveforms used in this study. Six co-field flow tubular chambers (*34*) 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. The enzyme solution was treated by PEF with alternating positive and negative pulses when bipolar pulses were applied. A cooling coil with a 2.3 mm inner diameter was connected to each pair of chambers and submerged in a water bath (model 1016, Fisher Scientific Inc., Pittsburgh, PA) to regulate temperature of enzyme solutions before and after PEF treatment. Type K thermocouples (Fisher Scientific, Pittsburgh, PA) were attached to the surface of the stainless steel coils near the inlet and outlet of each pair of PEF chambers. The temperatures of the inlet (T_1, T_3, and T_5)

and the outlet (T_2, T_4, and T_6) of each pair of chambers were monitored during PEF treatment by dual channel digital thermocouple readers (Fisher Scientific, Pittsburgh, PA). The places where thermocouples were located were isolated from atmosphere by an insulation tape (Polyethylene Cloth, Bron, Phoenix, AR). The initial temperatures of enzyme solution in the sample bank (T_0) and the sample temperature in the sample collecting bottle (T_7) were measured by mercury thermometers. A micro gear pump (model 020-000-010, Micropump, Inc., Vancouver, WA) maintained a continuous flow of enzyme solution. PEF treatment time (t) was calculated with the number of pulses received in the chambers (N_p), which is obtained from residence time in a chamber (T_r) as follows:

$$T_r = V/F \quad (1)$$

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

$$N_p = T_r \times f \quad (2)$$

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

$$t = N_p \times N_c \times W_p \quad (3)$$

where N_c is the number of treatment chambers and W_p is the pulse width (μs).

2.4. Heat Treatment. Pepsin solution (5 mL) was placed in a 25 × 200 mm glass test tube (Pyrex Borosilicate no. 48, Fisher Scientific) and heated to the desired temperature in a boiling water bath, and then rapidly cooled to 4 °C in an ice−water bath. A type K thermocouple (Fisher Scientific, Pittsburgh, PA) was inserted in the centered of the tube. The temperature of pepsin solution was monitored using a dual channel digital thermocouple reader (Fisher Scientific, Pittsburgh, PA). The tube was shaken by hand during the heating and cooling.

2.5. Measurement of Pepsin Activity. The activity of pepsin was measured by the method of Anson (*35*) with some modifications using hemoglobin as substrate. To prepare the 2.0% (w/v) hemoglobin substrate solution, 2.5 g of hemoglobin (Sigma-Aldrich Co., St. Louis, MO) was dissolved in 100 mL of distilled water and mixed vigorously, and then filtered with a glass wool filter. Before assay, 80 mL of filtrate was mixed with 20 mL of 300 mM HCl. Properly diluted enzyme solution (1.0 mL) was added to 5.0 mL of the above substrate solution which had been equilibrated in a 37 °C water bath for 20 min, mixed and incubated at 37 °C for exactly 10 min, and then 10 mL of 5.0% (W/V) TCA (Sigma Co., St. Louis, MO) was added and mixed vigorously for 1 min by a vortex (Genie 2, Fisher Scientific). The resultant mixture was filtered with a Waterman #50 filter paper (Sigma Co., St. Louis, MO). The filtrate was transfer to a quartz cuvette (Fisher Scientific, Pittsburgh, PA) to record the absorbance at 280 nm by a Spectronic Genesys 5 spectrometer (Milton Roy, Rochester, NY) at room temperature. The blank was prepared according to the above procedure, except that 1.0 mL of enzyme solution was replaced by 1.0 mL of distilled water. One unit was defined as a 0.001 increase of absorption unit at 280 nm per min by spectrometer.

2.6. Relative Residual Activity. The relative residual activity (RRA) of pepsin was defined as a percentage of activity of the PEF- or heat-treated pepsin solution to that of the control. For PEF treatment, the control went through the whole system in the same water bath temperature as it was during PET treatment, but without turning on PEF. For heat treatment, the control went through the system when the temperatures of both water baths were set 0 °C. Prior to activity assay, all samples were kept in a 0 °C ice−water bath.

2.7. Relative Protein Concentration. The relative protein concentration (RPC) of pepsin was defined as a percentage of the concentration of soluble protein of the PEF- or heat-treated pepsin solution to that of the control. RPC was calculated as follows:

$$\mathrm{RPC} = A_{280}/A_{C280} \quad (4)$$

where A_{280} is the absorbance of the PEF- or heat-treated pepsin solution filtered with a 0.45-μm syringe filter (Gelman Sciences, Ann Arbor, MI) and A_{C280} is the absorbance of the control filtered with the same model syringe filter. A_{280} and A_{C280} were measured by a Spectronic Genesys 5 spectrometer (Milton Roy) at room temperature.

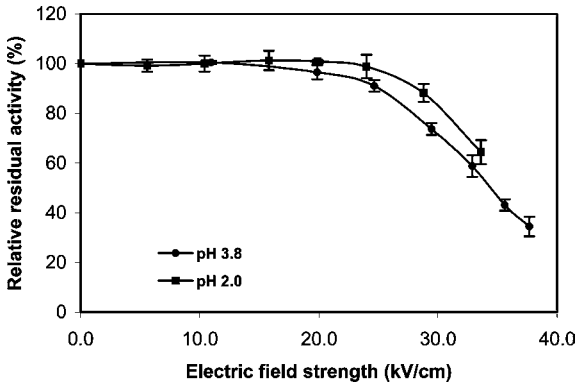

Figure 3. Inactivation of pepsin by PEF. The width and total treatment time were 2 and 126 μs, respectively. The media is 7.5 mM HCl with a pH of 2.0 and an electrical conductivity of 0.391 S/m and water−phosphate−acetate mixture with a pH of 3.8 and an electrical conductivity of 0.345 S/m.

2.8. First-Order Inactivation Kinetics of Pepsin by PEF. The experimental data were fit to the first-order inactivation models in eq 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).

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

where t is the PEF treatment time (μs) and k_N is the first-order kinetic constant.

2.9. UV Absorption Spectra. UV absorption spectra of pepsin solutions were recorded with a model UV-2401 PC UV−vis recording spectrophotometer (Shimadzu Scientific Instruments Inc, Columbia, MD) in a 1.0 cm quartz cuvette (Hellma, Muellheim, Baden, Germany) at 25 °C. Before scanning, all of the samples were filtered by a 0.45-μm syringe filter (Gelman Sciences, Ann Arbor, MI). The medium for each pepsin solution was used as its blank.

2.10. CD Analysis of Pepsin. Circular dichroism (CD) spectra were scanned at the far UV range (250−190 nm) with a model 202 circular dichroism spectrometer (AVIV Instruments Inc., Lakewood, NJ) in a 0.1 cm quartz CD cuvette (Hellma, Muellheim, Baden, Germany) at 25 °C. The concentration of protein was maintained at 3 μM. Deionized water was used as blank solution for all of the samples. The molar ellipticities of pepsin samples were calculated as follows:

$$[\theta] = \frac{S}{N \times C \times L \times 10} (\mathrm{deg\ cm^2\ dmol^{-1}}) \quad (6)$$

where S is the signal (mdeg) obtained by the circular dichroism spectrometer, N is the number of amino acids of pepsin molecule, C is the protein concentration (M) of the sample, and L is the cell path length (cm).

2.11. Relative Molar Ellipticity at 215 nm ($R[\theta]_{215\ nm}$). The relative molar ellipticity at 215 nm ($R[\theta]_{215nm}$) of pepsin was defined as a percentage of the molar ellipticity at 215 nm ($[\theta]_{215nm}$) of the PEF- or heat-treated pepsin to that of the control.

2.12. Statistical Analyses. All experiments were duplicated at least twice, and the assay of enzyme activity of each sample had been paralleled. The data presented are the means of each experiment. Analysis of variance was performed to determine variations among treatments.

3. RESULTS AND DISCUSSION

3.1. Effect of Electric Field Strength on the Activity of Pepsin. Figure 3 illustrates the effect of PEF treatment on the activity of pepsin in 7.5 mM HCl with a pH of 2.0 and an electrical conductivity of 0.391 S/m and water−phosphate−acetate mixture with a pH of 3.8 and an electrical conductivity

Table 1. Effect of Electric Field Strength on the Inactivation of Pepsin Activity

electric field strength (kV/cm)	relative residual activity compared to the control (%)	
	$T_2 - T_1 = 23$ °C	$T_2 - T_1 = 28$ °C
17.8	81.2 ± 2.17	63.6 ± 0.26
21.2	76.9 ± 0.83	62.4 ± 1.26
26.0	73.6 ± 1.84	57.5 ± 2.08
32.9	65.4 ± 0.71	39.9 ± 1.11

of 0.345 S/m. No change in pH was recorded after PEF treatment for each sample. Data in **Figure 3** show that PEF has a significant effect on the activity of pepsin and the activity reduction of pepsin was a function of applied electric field strength and pH. Pepsin is more stable in its optimum pH of 2.0 than in pH 3.8. To inactivate pepsin, the applied electric field strength or dosage should be beyond a certain level, such as 20 kV/cm for 126 μs in pH 3.8 and 25 kV/cm for 126 μs in pH 2.0. The significant increase of activities of pepsin after PEF treatment reported previously (*12*) was not observed. The contribution of heat produced by PEF treatment to the inactivation of pepsin is not significant (*22*). The highest temperature in all of the tests shown in **Figure 3** was lower than 52 °C. Pepsin is stable at 60 °C.

To explain the effect of the electric field strength on the inactivation of enzymes, further experiments were conducted with the change of temperature in each pair of PEF chambers kept constant, and the result is shown in **Table 1**. A temperature increase of enzyme solution with the same electrical conductivity is an indicator of the PEF dosage received by enzyme solution. To keep the temperature increase in each pair of chambers constant, the treatment time was changed with the change of applied electric field strength. It was achieved by adjusting the flow rate of enzyme solution. The data in **Table 1** show that relative residual activity of pepsin decreased with the increase of applied electric field strength under the condition of the same temperature increase. When the temperature increase in each pair of chamber was 23 and 28 °C, the relative residual activity of pepsin was decreased from 81.2% to 65.4% at 17.8 kV/cm and from 63.6% to 39.9% at 32.9 kV/cm, respectively. Higher electric field strength was more effective in inactivating pepsin; for example, the efficiency of PEF on inactivating enzymes is related to the electric field strength applied.

3.2. Effect of Electrical Conductivity on the Inactivation of Pepsin. In this set of experiment, the 1 mM HCl at pH 2.9 was used as the basic medium and the electric conductivity was adjusted by adding NaCl. 1 mM HCl at pH 2.9 was chosen as the basic medium for the study on the effect of electrical conductivity on the inactivation of pepsin by PEF. The electrical conductivity of 7.5 mM HCl at pH 2.0 is 0.345 S/m. Pepsin is highly stable at pH 2−4. **Figure 4** illustrates the effect of electrical conductivity on the efficiency of PEF on pepsin. The relative residual activity of pepsin steadily decreased with the increase of electrical conductivity at the same electric field strength for the same treatment time.

Most of the charged residues are distributed on the surface of a protein, and less than 5% are buried. Structural stability and functional properties of proteins are due to a delicate balance of a number of interactions, mainly noncovalent in nature. Among them, electrostatic interactions are factors (*36*). The electrostatic interactions are functions of pH, temperature, ion strength, and electric field. Higher electrical conductivity means more free ions in the solution. A high concentration of free ions

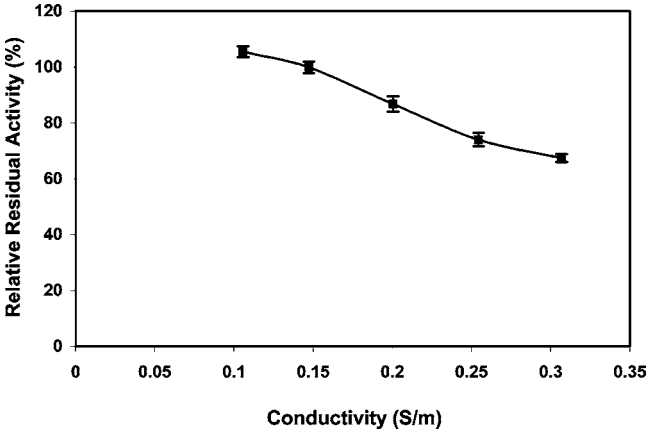

Figure 4. Influence of conductivity of solution on the effect of PEF on pepsin. The pH of the enzyme solution was 2.94. The pulse width and total treatment time were 2 and 126 μs, respectively. The applied electric field strength was 34.2 kV/cm.

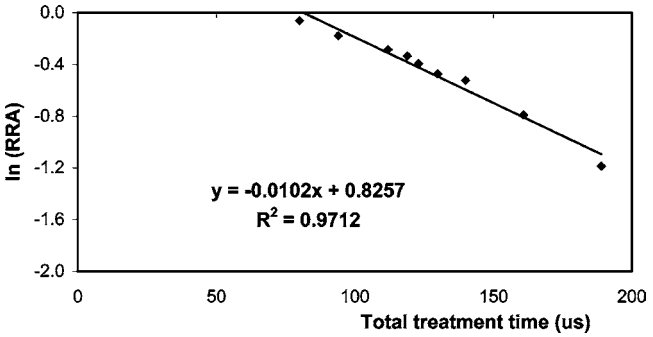

Figure 5. Schematic representation of the rate of inactivation of pepsin by PEF. The medium was 7.5 mM HCl with a pH of 2.0 and an electrical conductivity of 0.345 S/m. The pulse width was 2 μs. The applied electric field strength was 34.2 kV/cm.

may assist in suppressing or disrupting the electrostatic interactions under applied pulsed electric field.

3.3. Inactivation Kinetics of Pepsin by PEF. The effects of PEF treatment time on the RRA of pepsin in 7.5 mM HCl at pH 2.0 and electrical conductivity of 0.345 S/m at 34.2 kV/cm are shown in **Figure 5**. The RRA values as a function of PEF treatment time were fit to the first-order inactivation model in eq 5 when the total treatment time is higher than 80 μs. The correlation coefficient (r^2) of the first-order inactivation model was 0.9717. It suggests that the first-order inactivation model may be suitable for describing the inactivation of pepsin 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 pepsin in 7.5 mM HCl at pH 2.0 and electrical conductivity of 0.345 S/m at 34.2 kV/cm was 0.012 (1/μs). The trend line of ln(RRA) versus treatment time (t) suggests that the first-order inactivation kinetics is valid when, at 34.2 kV/cm, the treatment time is longer than 80 μs. When the treatment time was 80 μs, the RRA was 94.1%. It indicates that to inactivate pepsin, PEF dosage should be higher than a critical level, which may be defined as critical dosage for the inactivation of pepsin by PEF.

3.4. Effect of PEF Treatment on the Solubility of Pepsin. In the study on the effect of PEF on the solubility and secondary structure of pepsin, water−phosphate−acetate mixture at pH 3.8 and an electrical conductivity of 0.345 S/m were used to avoid using HCl. Chloride ion is a strong absorbent in the UV range and will interrupt the measurement of the CD signal of pepsin. **Figure 6** illustrates the effect of PEF treatment on the

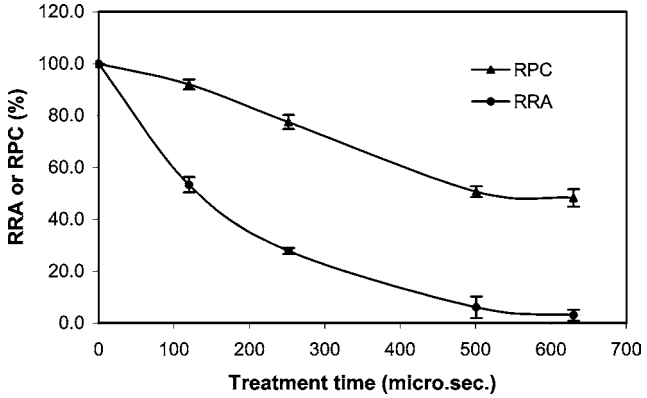

Figure 6. Effect of PEF treatment on the activity and solubility of pepsin in water–phosphate–acetate mixture with a pH of 3.8 and an electrical conductivity of 0.345 S/m. The concentration of total protein including the soluble and insoluble in the control and PEF-treated samples was 86.6 μM.

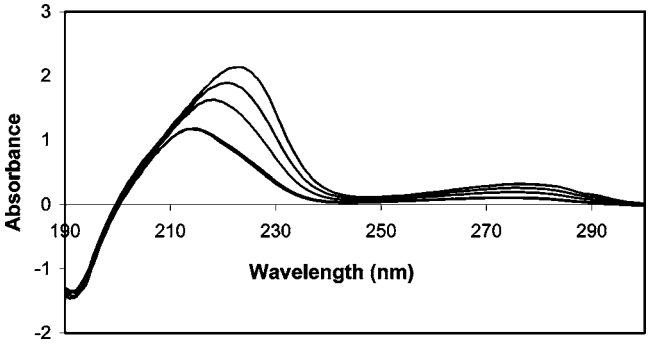

Figure 7. UV spectra of pepsin in a medium of water–phosphate–acetate at pH 3.8 and electrical conductivity of 0.334 S/m at 25 °C before and after PEF treatment. The pulse width and pulse repetition rate were 2 μs and 800 pps, respectively. The applied electric field strength was 35.6 kV/cm. Curves from top to bottom correspond to test results with PEF treatment at 35.6 kV/cm for 0, 126, 252, 504, and 603 μs, respectively.

solubility and activity of pepsin in water−phosphate−acetate mixture at pH 3.8 and an electrical conductivity of 0.345 S/m. When the concentration of total protein including the soluble and insoluble was 86.6 μM, both activity and solubility decreased with the increase of treatment time. Activity decreased much more rapidly than solubility did. This phenomenon suggests that PEF treatment may cause the aggregation of pepsin protein, but the inactivation occurred before the protein formed aggregates. When the concentration of pepsin protein was much lower, for instance at 3.1 μM (**Figure 3**), no significant aggregation was observed, but significant activity reduction was observed.

Figure 7 shows the UV spectra of untreated and PEF-treated pepsin in water−phosphate−acetate mixture at pH 3.8 and an electrical conductivity of 0.345 S/m. With an increase of the PEF treatment time, the absorbance peaks near 280 nm did not significantly shift, but the absorbance peaks near 220 nm shifted toward lower wavelength from 223 nm of control to 213.5 nm of the sample treated at 35.6 kV/cm for 730 μs. The decrease of the height of the peaks with the increase of the treatment time was caused by the aggregation of pepsin by PEF treatment, which is in agreement with the result shown in **Figure 6**.

3.5. Effect of PEF Treatment on the Secondary Structure of Pepsin. Circular dichroism spectra can directly interpret the changes of protein secondary structure (*37*−*41*). The CD analysis was used to detect the change of the secondary structure

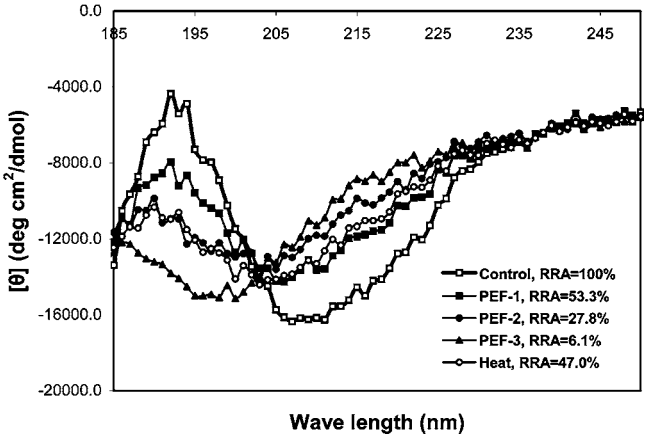

Figure 8. Far-UV CD spectra of pepsin before and after PEF or heat treatment in water–phosphate–acetate mixture at pH 3.8 and electrical conductivity of 0.334 S/m. For PEF treatment, the pulse width and pulse repetition rate were 2 μs and 800 pps, respectively. The applied electric field strength was 35.6 kV/cm. The treatment times of PEF-1, PEF-2, and PEF-3 were 126, 252, and 630 μs, respectively. The heat treatment was conducted in a boiling water bath. Pepsin solution of 5 mL in a tube was heated to 74 °C from 4 °C and then rapidly cooled to 4 °C in an ice–water bath.

of pepsin after PEF treatment and heat treatment. Pepsin is a β-sheet dominant protein (*42*, *43*). **Figure 8** shows the far-UV CD spectra of pepsin before and after PEF or heat treatment. The spectrum of the control has a typical shape of a β-sheet-rich protein, in agreement with the measurement by Konno and others (*33*). It is not identical to that of pepsin in its native state. The spectrum of pepsin in its native state has a single minimum at 210−215 nm, which is typical for a very highly β-sheet-rich protein (*33*, *44*). It means the secondary structure of the pepsin used as the control may has been slightly changed during purification and storage. With the increase of PEF treatment time, the minima of the CD spectra of the PEF-treated pepsin shifted to a lower wavelength region, and the intensity of the negative peak between 195 and 220 nm decreased. It indicates a gradual loss of β-sheet structure and the emergence of intensity characteristic of random coil regions of structure (*45*−*47*) with an increase of PEF treatment under the tested condition. The heat treatment also caused the loss of the β-sheet structure of pepsin.

Acid proteinases have a characteristic minimum at 215 nm (*48*−*51*). The increase of molar ellipticity at 215 nm means the loss of the second structure of pepsin, featuring a very high β-sheet content. **Figure 9** shows the relationship between relative residual activity (RRA) and the residue molar ellipticity at 215 nm ($[\theta]_{215nm}$) of PEF-treated pepsin as compared to the control. The results show that the decrease of RRA is inversely related to the increase of $[\theta]_{215nm}$. It indicates that inactivation of pepsin is related to the loss of the second structure.

Table 2 further illustrates the relationship between relative residual activity and the relative molar ellipticity at 215 nm. The RRA decreased with the decrease of $[\theta]_{215nm}$ in both the PEF-treatment and the heat-treatment test. The ratio of RRA/$[\theta]_{215nm}$ was not kept constant with the change of RRA. It decreased with the decrease of RRA and $[\theta]_{215nm}$. It means the activity of pepsin changed more quickly than its secondary structure. When the RRA values were similar, such as 27.8% in PEF treatment and 29.2% in heat treatment, the RRA/$[\theta]_{215nm}$ values were also similar, 0.409 in PEF treatment and 0.414 in heat treatment. It indicates that the degree of inactivation of pepsin is a function of the degree of change in its secondary

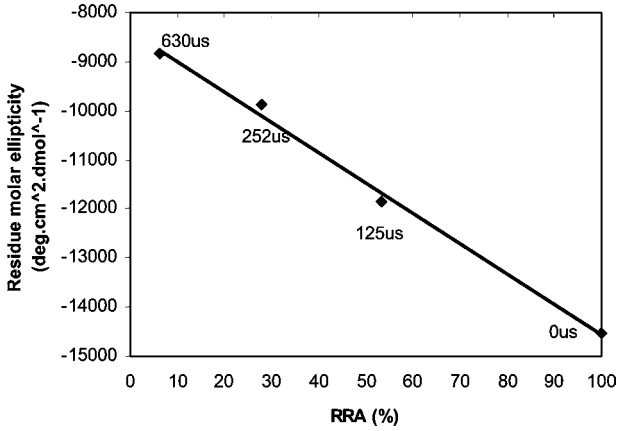

Figure 9. Treatment-dependent changes in relative residual activity (RRA) and the residue molar ellipticity at 215 nm ($[\theta]_{215nm}$). The medium was water–phosphate–acetate mixture at pH 3.8 and an electrical conductivity of 0.334 S/m. The pulse width and pulse repetition rate were 2 μs and 800 pps, respectively. The applied electric field strength was 35.6 kV/cm.

Table 2. RRA and $R[\theta]_{215nm}$ of PEF- or Heat-Treated Pepsin in a Water–Phosphate–Acetate Mixture with a pH of 3.8 and an Electrical Conductivity of 0.334 S/m

treatment	RRA (%)	$R[\theta]_{215nm}$ (%)	RRA/$R[\theta]_{215nm}$
PEF-1[a]	53.3	81.6	0.653
PEF-2[a]	41.7	74.5	0.560
PEF-3[a]	27.8	68.0	0.409
heat-1[b]	47. 0	78.0	0.603
heat-2[b]	29.2	70.5	0.414

[a] PEF represents PEF treatment. The pulse width and pulse repetition rate were 2 μs and 800 pps, respectively, and the applied electric field strength was 35.6 kV/cm. [b] Heat represents heat treatment conducted in a boiling water bath.

structure. The inactivation of pepsin, no matter whether it is achieved by PEF or heat treatment, is the result of the loss of its secondary structure.

Yeom et al. (*14*) observed loss of α-helix structure in PEF-treated papain with the significant reduction of papain activity. Neumann and Katchalsky (*52*) 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 (*53*). Yeom et al. (*14*) explained α-helix structure was susceptible to conformational by high electric field due to its dipole moment. Each peptide bond carries a dipole moment, and the alignment of peptide bonds may give rise to a macrodipole of considerable strength (*54*, *55*). There is a lack of reports on the effect on β-sheet structure.

3.6. Conclusions. The activity reduction of PEF-treated pepsin was a function of applied electric field strength, PEF treatment time, electrical conductivity, and pH. The first-order inactivation model was valid for describing the inactivation of pepsin by PEF under the applied electric field strength. The first-order inactivation kinetic constant k_N of pepsin in 7.5 mM HCl at pH 2.0 and an electrical conductivity of 0.345 S/m at 34.2 kV/cm was 0.012 (1/μs). Aggregation of pepsin by PEF treatment was observed. The inactivation of pepsin occurred before the aggregates were formed. The disruption of the β-sheet structure was observed in PEF-treated pepsin by CD analysis. Inactivation of PEF-treated pepsin was related to the loss of native secondary structure featuring a very high β-sheet content. The relative residual activity (RRA) of PEF-treated pepsin was related to the residue molar ellipticity at 215 nm ($[\theta]_{215nm}$), but the ratio of RRA/$[\theta]_{215nm}$ was not kept constant with the change of RRA. The ratio decreased with the decrease of RRA and $[\theta]_{215nm}$. Both PEF and heat achieved inactivation by alternating the secondary structure of pepsin.

ACKNOWLEDGMENT

Dr. G. Renkes at the Department of Chemistry of The Ohio State University is gratefully acknowledged for his help in operating the AVIV 202 circular dichroism spectrometer.

LITERATURE CITED

(1) Barbosa-Canovas, G. V.; Pierson, M. D.; Zhang, Q. H.; Schaffner, D. W. Pulsed electric fields. *J. Food Sci.* **2000**, (Spec. Suppl., Kinetics of Microbial Inactivation for Alternative Food Processing Technologies), 65−81.

(2) Min, S.; Jin, Z. T.; Min, S. K.; Yeom, H.; Zhang, Q. H. Commercial-scale pulsed electric field processing of orange juice. *J. Food Sci*. **2003**, *68*, 1265−1271.

(3) Min, S.; Jin, Z. T.; Zhang, Q. H. Commercial Scale Pulsed Electric Field Processing of Tomato Juice. *J. Agric. Food Chem.* **2003**, *51*, 3338−3344.

(4) Mertens, B.; Knorr, D. Developments of nonthermal processes for food preservation. *Food Technol.* **1992**, *46*, 125−133.

(5) Knorr, D. Novel approaches in food-processing technology: new technologies for preserving foods and modifying function. *Curr. Opin. Biotechnol.* **1992**, *10*, 485−91.

(6) Jin, Z. T.; Zhang, Q. H. Pulsed electric field inactivation of microorganisms and preservation of quality of cranberry juice. *J. Food Process. Preserv.* **1999**, *23*, 481−487.

(7) Yeom, H. W.; Streaker, C. B.; Zhang, Q. H.; Min, D. B. Effects of pulsed electric fields on the activities of microorganisms and pectin methyl esterase in orange juice. *J. Food Sci.* **2000**, *65*, 1359−1363.

(8) Evrendilek, G. A.; Jin, Z. T.; Ruhlman, K. T.; Qiu, X.; Zhang, Q. H. Microbial safety and shelf life of apple juice and cider processed by bench and pilot scale PEF systems. *Innovative Food Sci. Emerging Technol*. **2000**, *1*, 77−86.

(9) Ayhan, Z.; Yeom, H. W.; Zhang, Q. H.; Min, D. B. Flavor, Color, and Vitamin C Retention of Pulsed Electric Field Processed Orange Juice in Different Packaging Materials. *J. Agric. Food Chem.* **2001**, *49*, 669−674.

(10) Ayhan, Z.; Zhang, Q. H.; Min, D. B. Effects of pulsed electric field processing and storage on the quality and stability of single-strength orange juice. *J. Food Prot.* **2002**, *65*, 1623−1627.

(11) Li, S. Q.; Zhang, Q. H.; Lee, Y. Z.; Pham, T. V. Effects of pulsed electric fields and thermal processing on the stability of bovine IgG (IgG) in enriched soymilk. *J. Food Sci*. **2003**, *68*, 1201−1207.

(12) Ho, S. Y.; Mittal, G. S.; Cross, J. D. Effect of high field electric pulses on the activity of selected enzymes. *J. Food Eng.* **1997**, *31*, 69−84.

(13) Van Loey, A.; Verachtert, B.; Hendrichx, M. Effects of high electric field pulses on enzymes. *Trends Food Sci. Technol.* **2002**, *12*, 94−102.

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

(15) Giner, J.; Gimeno, V.; Barbosa-Canovas, G. V.; Martin, O. Effects of pulsed electric field processing on apple and pear polyphenoloxidases. *Food Sci. Technol. (London)* **2001**, *7*, 339−345.

(16) Giner, J.; Ortega, M.; Mesegue, M.; Gimeno, V.; Barbosa-Canovas, G. V.; Martin O. Inactivation of peach polyphenoloxidase by exposure to pulsed electric fields. *J. Food Sci.* **2002**, *67*, 1467−1472.

(17) Grahl, T.; Märkl, H. Killing of microorganisms by pulsed electric fields. *Appl. Microbiol. Biotechnol.* **1996**, *45*, 148−57.

(18) Castro, A. J.; Swanson, B. G.; Barbosa-Canovas, G. V.; Zhang, Q. H. Pulsed electric field modification of milk alkaline phosphatase activity. In *Pulsed Electric Fields in Food Processing*; Barbosa-Canovas, G. V., Zhang, Q. H., Tabilo-Munizaga, G., Eds.; Technomic Publishing Co., Inc.: Lancaster, PA, 2001; pp 65−82.

(19) Vega-Mercado, H.; Powers, J. R.; Barbosa-Canovas, G. V.; Swanson, B. G. Plasmin inactivation with pulsed electric fields. *J. Food Sci.* **1995**, *60*, 1143−1146.

(20) Vega-Mercado, H.; Powers, J. R.; Martin-Belloso, O.; Luedecke, L.; Barbosa-Canovas, G. V.; Swanson, B. G. Changes in susceptibility of proteins to proteolysis and the inactivation of an extracellular protease from *Pseudomonas fluorescens* M3/6 when exposed to pulsed electric fields. In *Pulsed Electric Fields in Food Processing*; Barbosa-Canovas, G. V., Zhang, Q. H., Tabilo-Munizaga, G., Eds.; Technomic Publishing Co., Inc.: Lancaster, PA, 2001; pp 105−120.

(21) Bendicho, S.; Barbosa-Canovas, G. V.; Martin, O. Reduction of protease activity in milk by continuous flow high-intensity pulsed electric field treatments. *J. Dairy Sci.* **2003**, *86*, 697−703.

(22) Yang, R. J.; Li, S. Q.; Zhang, Q. H. Effects of Pulsed Electric Fields on the Activity of Enzymes in Aqueous Solution. *J. Food Sci*. **2004**, *69*, FCT241−248.

(23) Lipatov, N. N.; Boreskov, V. G.; Krokha, N. G.; Mitaseva, L. F.; Stefanov, A. V. Biotechnological modification of meat trimmings with a high level of connective tissue. *Izv. Vyssh. Uchebn. Zaved., Pishch. Tekhnol.* **1989**, *5*, 12−15.

(24) Kodama, K.; Morimatsu, F.; Uto, Y.; Kimura, S. Nippon Meat Packers, Japan. Jpn. Kokai Tokkyo Koho, Protease-treated pork products. JP Patent 05030944, 1993.

(25) Barbano, D. M.; Rasmussen, R. R. Cheese yield performance of fermentation-produced chymosin and other milk coagulants. *J. Dairy Sci.* **1992**, *75*, 1−12.

(26) Ohtani, H. Coagulated milk and enzymes. *New Food Ind.* **1988**, *30*, 65−79.

(27) Champagne, E. T.; Hinojosa, O.; Clemetson, C. A. B. Production of ascorbate free radicals in infant formulas and other media. *J. Food Sci*. **1990**, *55*, 1133−6.

(28) Tomita, M.; Wakabayashi, H.; Yamauchi, K.; Teraguchi, S.; Hayasawa, H. Bovine lactoferrin and lactoferricin derived from milk: production and applications. *Biochem. Cell Biol.* **2002**, *80*, 148.

(29) Pitts, J. E.; Dhanaraj, V.; Dealwis, C. G.; Mantafounis, D.; Nugent, P.; Orprayoon, P.; Cooper, J. B.; Newman, M.; Blundell, T. L. *Scand. J. Clin. Lab. Invest*. **1992**, *52*, 39−50.

(30) Antonov, V. K.; Ginodman, L. M.; Kapitannikov, Y. V.; Barshevskaya, T. N.; Gurova, A. G.; Rumsh, L. D. *FEBS Lett.* **1978**, *88*, 87−90.

(31) Cornish-Bowden, A. J.; Knowles, J. R. The pH-dependence of pepsin-catalysed reactions. *Biochem. J.* **1969**, *113*, 353−362.

(32) McPhie, P. A reversible unfolding reaction of swine pepsin; implications for pepsinogen's folding mechanism. *Biochem. Biophys. Res. Commun.* **1989**, *158*, 115−119.

(33) Konno, T.; Kamatari, Y. O.; Tanaka, N.; Kamikubo, H.; Dobson, C. M.; Nagayama, K. A partially unfolded structure of the alkaline-denatured state of pepsin and its implication for stability of the zymogen-derived protein. *Biochemistry* **2000**, *39*, 4182−4190.

(34) Yin, Y.; Zhang, Q. H.; Sastry, S. K. High voltage pulsed electric field treatment chambers for the preservation of liquid food products. U.S. Patent 5,690,978, 1997.

(35) Anson, M. L. The estimation of pepsin, typsin, papain, and cathepsin with hemoglobin. *J. Gen. Physiol.* **1938**, *22*, 79−89.

(36) Koumanov, A.; Ladenstein, R.; Karshikoff, A. Electrostatic interactions in proteins: Contribution to structure−function relationships and stability. *Recent Res. Dev. Protein Eng.* **2001**, *1*, 123−148.

(37) Townend, R.; Kumosinski, T. F.; Timasheff, S. N.; Fasman, G. D.; Davidson, B. The circular dichroism of the beta structure of poly-L-lysine. *Biochem. Biophys. Res. Commun.* **1966**, *23*, 63−9.

(38) Hennessey, J. P., Jr.; Johnson, W. C., Jr. Information content in the circular dichroism of proteins. *Biochemistry* **1981**, *20*, 1085−94.

(39) Venyaminov, S. Y.; Yang, J. T. Determination of protein secondary structure. In *Circular Dichroism and the Conformational Analysis of Biolecules*; Fasman, G. D., Ed.; Plenum Press: New York and London, 1996; pp 69−107.

(40) Johnson, W. C. Analyzing protein circular dichroism spectra for accurate secondary structures. *Proteins* **1999**, *35*, 307−12.

(41) Shellman, J. A. Circular dichroism and optical rotation. *Chem. Rev.* **1975**, *75*, 323−331.

(42) James, M. N.; Sielecki, A. R. Molecular structure of an aspartic proteinase zymogen: porcine pepsinogen, at 1.8 A resolution. *Nature* **1986**, *319*, 33−38.

(43) Abad-Zapatero, C.; Rydel T. J; Erickson, J. Revised 2.3 A structure of porcine pepsin: evidence for a flexible subdomain. *Proteins* **1990**, *8*, 62−81.

(44) Kamatari, Y. O.; Dobson, C. M.; Konno, T. Structural dissection of alkaline-denatured pepsin. *Protein Sci.* **2003**, *12*, 717−724.

(45) Bovey, F. A.; Yanari, S. S. In *The Enzyme III*; Boyer, P. D., Ed.; Academic Press: New York, 1960; pp 63−91.

(46) Sielecki, A. R.; Fedorov, A. A.; Boodhoo, A.; Andreeva, N. S.; James, M. N. Molecular and crystal structures of monoclinic porcine pepsin refined at 1.8 A resolution. *J. Mol. Biol*. **1990**, *214*, 143−170.

(47) Favilla, R.; Parsoli, A.; Mazzini, A. Alkaline denaturation and partial refolding of pepsin investigated with DAPI as an extrinsic probe. *Biophys. Chem*. **1997**, *67*, 75−83.

(48) Tello-Solís, S. R.; Hernandez-Arana, A. Effect of irreversibility on the thermodynamic characterization of the thermal denaturation of *Aspergillus saitoi* acid proteinase. *Biochem. J.* **1995**, *311*, 969−974.

(49) Tello-Solís, S. R.; Rodriguez-Romero, A.; Hernandez-Arana, A. Circular dichroism studies of acid proteinases from *Aspergillus niger* and Aspergillus awamori. *Biochem. Mol. Biol*. **1994**, *33*, 759−768.

(50) Allen, B.; Blum, M.; Cunningham, A.; Tu, G. C.; Hofmann, T. A ligand-induced, temperature-dependent conformational Change in penicillopepsin: Evidence from nonlinear Arrhenius plots and from circular dichroism studies. *J. Biol. Chem.* **1990**, *265*, 5060−5065.

(51) Tello-Solís, S.; Romero-García, B. Thermal denaturation of porcine pepsin: a study by circular dichroism. *Int. J. Biol. Macromol.* **2001**, *28*, 129−133.

(52) Neumann, E.; Katchalsky, A. Long-lived conformation changes induced by electric pulses in biopolymers. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 993−997.

(53) Tsuji, Y.; Yasunaga, T.; Sano, T.; Ushio, H. Kinetic studies of the Helix-coil transition in aqueous solutions of poly (α-L-glutamic acid) using the electric field pulse method. *J. Am. Chem. Soc*. **1976**, *98*, 813−818.

(54) Wada A. Chain regularity and dielectric properties of poly-α-amino acids in solution. In *Polyamino Acids, Polypeptides, and Proteins*; Stahman, M. A., Ed.; The University of Wisconsin Press: Madison, WI, 1962; pp 31−146.

(55) Wada A. The α-helix as an electric macro-dipole. *Adv. Biophys.* **1976**, *9*, 1−63.

Received for review May 20, 2004. Revised manuscript received September 7, 2004. Accepted September 15, 2004. We acknowledge funding support from The U.S. Army Natick Soldier System Center and personal support from the China Scholarship Council.

JF049183P