Effects of Pulsed Electric Fields on the Activity of Enzymes in Aqueous Solution

R.-J. YANG, S.-Q. LI, AND Q.H. ZHANG

ABSTRACT: A group of selected enzymes were subjected to continuous pulsed electric field (PEF) treatments to evaluate the inactivation effect of PEF. For a treatment time of 126 μs, 51.7% and 83.8% of pepsin was inactivated at 37.0 kV/cm and 41.8 kV/cm, respectively. Enzyme activity of polyphenol oxidase decreased 38.2% when treated at 33.6 kV/cm for 126 μs. Enzyme activity decreased 18.1% and 4.0% for peroxidase treated at 34.9 kV/cm and chymotrypsin treated at 34.2 kV/cm, respectively. No significant change in lysozyme activity was observed after PEF from 0 to 38 kV/cm for 126 μs. Both PEF and the induced heat contributed to the observed inactivation effect, depending on the properties of enzymes and test conditions.

Keywords: pulsed electric fields, enzyme inactivation, field strength, total treatment time, electrical conductivity

Introduction

High-voltage pulsed electric fields (PEF) is an emerging non-thermal food-preservation technology, which has been researched and developed close to commercial stage (Barbosa-Canovas and others 2000; Zhang and others 2002; Min and others 2003a, 2003b). Compared with thermal processing, PEF has a number of advantages including minimal changes of flavor, taste, color, nutrients (Mertens and Knorr 1992; Knorr 1999; Ayhan and others 2001, 2002), and functionality (Li and others 2003). Therefore, PEF is receiving considerable attention from the food industry. Many studies investigated the inactivation of microorganisms by PEF and effects on quality and shelf-life of foods. PEF is effective in inactivating a wide range of microorganisms at ambient temperature. Little impact on the flavor of the treated foods compared with heat treatment was observed (Yeom and others 2000b).

Inactivation of both microorganisms and enzymes is critical in food processing and preservation. However, compared with the extensive studies on the inactivation of microorganisms by PEF, there are limited reports about the effects of PEF on enzymes. Moreover, the conclusions about the effects of PEF on enzyme inactivation from different research groups are inconsistent.

Ho and others (1997) reported that the sensitivity of enzymes to PEF varied from enzyme to enzyme. Under the tested conditions (13 to 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% to 85% after PEF treatment. Peroxidase and polyphenol oxidase showed a moderate reduction of 30% to 40%. Alkaline phosphatase activity showed only a slight reduction of 5%. Pepsin activity was not decreased but significantly increased at 20 kV/cm to about 45 kV/cm. Van Loey and others (2002) studied effects of mono-polar square-wave high electric field pulses on several enzymes using flexible laboratory-scale equipment and reported that lipoxygenase, polyphenol oxidase, pectin-methylesterase, and peroxidase are resistant to PEF treatment in distilled water but sensitive in more complex liquid dairy or fruit and vegetable products. Van Loey and others (2002) suggested that the inactivation of enzymes by PEF is actually caused by thermal effects. Yeom and others (1999) studied inactivation of heat-stable papain by PEF in a continuous system and reported that irreversible reduction of activity was observed in PEF-treated papain, and the activity was not recovered after 24 h storage at 4 °C. Yeom and others (1999) suggested that the inactivation of papain is because of the loss of α-helix structure in papain molecules, and heat contributes minimally to the observed inactivation effect. Yeom and others (2000a) investigated the effects of PEF on pectin methyl esterase (PME) activity in a pilot-plant system and observed that about 90% of PME activity was inactivated by PEF treatment at 35 kV/cm for 59 μs. Significant activity reductions of polyphenol oxidase after PEF treatment with bipolar exponential decay pulses, 97% in apple extract at 24.6 kV/cm for 6000 μs, 72% in pear extract at 22.3 kV/cm for 6000 μs (Giner and others 2001), and 70% in peach extract at 24.30 kV/cm for 5000 μs (Giner and others 2002) were reported. Grahl and Märkl (1996) investigated alkaline phosphatase, lactoperoxidase, and lipase in raw milk using a batch PEF device with the chambers composed of 2 parallel carbon electrodes (A = 50 cm^2, d = 0.5 cm) and reported an activity reduction of lipase of 60% at an energy input of 200 kJ/L but no inactivation of alkaline phosphatase and lactoperoxidase after PEF treatment of 21.5 kV/cm and a total energy input of 400 kJ/L. However, the research conducted by Castro and others (2001) observed 65% activity reduction of alkaline phosphatase in raw milk after PEF treatment at 18.8 kV/cm with 70 pulses. Vega-Mercado and others investigated the effect of PEF on plasmin in simulated milk ultra-filtrate (Vega-Mercado and others 1995a) and an extracellular protease from *Pseudomonas fluorescens* M3/6 in 3 media (Vega-Mercado and others 1995b, 2001) using a continuous treatment chamber (gap = 0.6 cm) and reported that plasmin activity decreased 90% after PEF treatment with 50 pulses of 2 μs at 30 and 45 kV/cm. Eighty percent of activity reduction was achieved in tryptic soy broth with yeast extract after 20 pulses of 2 μs at 18 kV/cm. No significant inactivation of protease in casein-Tris buffer was observed after PEF treatment. However, the proteolytic activity of protease in skim milk after PEF treatment at 25 kV/cm and 0.6 Hz increased. These results indicate that environmental conditions might influence the sensitivity of enzymes to PEF. Bendicho and

MS 20030506 Submitted 9/5/03, Revised 10/21/03, Accepted 1/12/04. Author Yang is with School of Food Science and Technology, Southern Yangtze Univ., 170 Huihe Rd., Wuxi, Jiangsu 214036, P.R. China. Authors Li and Zhang are with the Dept. of Food Science and Technology, The Ohio State Univ., 2015 Fyffe Rd., 110 Parker Food Science and Technology Building, Columbus, OH 43210. Direct inquiries to author Zhang (E-mail: zhang.138@osu.edu).

others (2003) investigated the effectiveness of PEF on inactivation of a protease from *Bacillus subtilis* inoculated in milk using 8 colinear chambers connected in series in a continuous PEF equipment and reported that protease activity decreased with increased treatment time or field strength and pulse repetition rate. Milk composition affected the results and higher inactivation levels were reached in skim milk than in whole milk. The maximum inactivation (81%) was attained in skim milk after an 866-μs treatment at 35.5 kV/cm and 111 Hz. No differences were observed between 4- and 7-μs pulse widths when total treatment time was kept the same.

Different research groups used different PEF devices and different treatment conditions. The results about the effects of PEF on enzymes from different research groups are inconsistent. Although stainless steel was chosen as electrode materials in almost all of the studies on inactivation of enzymes by PEF, the geometry of the treatment chambers and electrodes vary from parallel plate, coaxial cylinder to cofield. It affects the distribution of electric field and the dosage taken by enzyme in different location of the chamber. The wave shape used varies from exponential decay to square wave, where pulse width may be measured at 37% peak and 50% peak, respectively. Polarity of the pulse may take either bipolar or monopolar. The variation in media, such as the variety of electrolytes and the ion strength and pH, has effects on the stability of enzymes. Different electrical conductivities of the media will result in different temperature gradient with the same electric field strength and treatment time. The pulse frequency and cooling system before and after treatment chambers also affects the treatment temperature. The temperature control/registration during PEF treatment is often not considered or reported in details so that no distinction can be made between thermal and PEF effects on enzymes. It is necessary to take into account all of the PEF system and experimental conditions, such as electric field strength, number of pulses, wave shape pulse width and batch, and stepwise or continuous circulation, in making a comparison between different studies.

The majority of published work was conducted with either batch process using parallel electrodes or continuous process using coaxial electrodes. Reported total treatment time is much longer than the practical range (<200 μs). Furthermore, although inactivation of some food-related enzymes by PEF has been investigated and reported, the mechanism involved is not clear. The objectives of this study were (1) to investigate the inactivation of enzymes by a continuous PEF processor with cofield flow treatment chambers, which has been developed close to commercial stage at the Ohio State Univ., (2) to evaluate the effects of environment conditions such as pH, electrical conductivity, heat induced during PEF treatment, and temperature, and (3) to find the primary variable responsible for the inactivation of enzymes by PEF.

Materials and Methods

Materials

Five enzymes, pepsin, lysozyme, peroxides, chymotrypsin, and polyphenol oxidase, were selected based on their size, structure, cofactors, and properties for this study. Pepsin, lysozyme, peroxides, and chymotrypsin are monomers, whereas polyphenol oxidase from mushroom is a tetramer with a molecular weight of 128 kD (Jolley and others 1969a, 1969b). The thermostability of peroxidase is much higher than that of the other 4 enzymes. Peroxidase and polyphenol oxidase are metal-containing enzymes, and Fe^{3+} and Cu^{2+} (Brooks and Dawson 1966) are essential for their activities, respectively. Pepsin has a very low optimum pH of 2, and lysozyme has a low molecular weight of 14.3 kD.

Table 1—Selection of media for each enzyme

Enzyme	Media	Enzyme concentration
Pepsin	7 m*M* HCl	70 to 90 Units/mL; 0.6 to 0.8 μmol protein/mL
	1 m*M* HCl + NaCl[a]	70 to 90 Units/mL
	10 m*M* potassium phosphate + NaCl[a]	70 to 90 Units/mL
Lysozyme	10 m*M* potassium phosphate + NaCl[a]	750 to 1000 Units/mL 15 to 20 μg protein/mL
Peroxidase	10 m*M* potassium phosphate + NaCl[a]	10 to 13 Units/mL; 80 to 100 μg/mL
Polyphenol oxidase	10 m*M* potassium phosphate + NaCl[a]	150 to 200 μg/mL; 300 to 400 units/mL
Chymotrypsin	10 m*M* potassium phosphate + NaCl[a]	3 to 4 Units/mL; 70 to 90 μg protein/mL

[a]NaCl was used to adjust the electrical conductivity.

Pepsin (P-6887, from porcine stomach mucosa, purified by crystallization followed by chromatography, essentially salt-free), peroxidase (P-1432, from soybean, essentially salt-free powder), lysozyme (L-6876, from chicken egg white, 3 × crystallized, dialyzed, and lyophilized, approximately 95% protein), polyphenol oxidase (T-7755, from mushroom), α-chymotrypsin (C-4129, from bovine pancreas, 3 × crystallized from 4 × crystallized chymotrypsinogen, dialyzed essentially salt-free and lyophilized powder), and other chemicals were purchased from Sigma Co. (St. Louis, Mo., U.S.A.).

Preparation of enzyme solutions

Enzymes were dissolved in cooled (4 °C to 6 °C) media with a selected pH and electrical conductivity just before PEF or heat treatment. The media for tested enzymes are listed in Table 1. The enzyme concentration was based on the activity of the pure enzymes purchased from Sigma. The concentrations of all enzyme solutions for this study of were within the values recommended by Sigma for activity assays.

PEF treatment

A bench-scale continuous PEF system (OSU-4A, The Ohio State Univ., Columbus, Ohio, U.S.A.) was used to treat the enzyme solutions. A schematic diagram is shown in Figure 1. A model 9310 trigger generator (Quantum Composer Inc., Bozeman, Mont., U.S.A.) was used to control replication rate and duration time of pulses. Voltage, current, frequency, and waveform were monitored by a 2-channel 1-GS/s (60 MHz bandwidth) digital real-time oscilloscope (Model TDS 210, Tektronix Inc., Wilsonville, Ore., U.S.A.). Figure 2 shows a typical set of voltage and current waveforms used in this study. Six cofield flow tubular chambers (Yin and others 1997) with a 2.92-mm electrode gap and a 2.3-mm inner dia were grouped in 3 pairs, and each pair were connected with stainless-steel tubing with a 2.3-mm inner dia. The enzyme solutions were alternatively treated by positive and negative high-voltage pulses, when bipolar pulses were applied. A 95-cm-long stainless-steel tube with a 2.3-mm inner dia was connected to each pair of chambers, about 65 cm of the tube was coiled and submerged in a water bath (Model 1016, Fisher Scientific Inc., Pittsburgh, Pa., U.S.A.) to cool enzyme solutions after PEF treatment. Type K thermocouples (Fisher Scientific) were attached to the surface of the stainless-steel coils, 2.5 cm away from the PEF zones along the flow direction. The temperatures of inlet (T_1, T_3, and T_5) and outlet (T_2, T_4, and T_6) of the chambers

were monitored during PEF treatment by dual-channel digital thermocouple readers (Fisher Scientific). The places where thermocouples were located were isolated from atmosphere by an insulation tape (Polyethylene Cloth, Bron, Phoenix, Ariz., U.S.A.). The temperatures were recorded when the readings were stable. The distance between the chamber and the nearby inlet or outlet temperature checkpoint was about 2.5 cm. The distance between the temperature checkpoint and the surface of cooling water was 15 cm. When the total treatment was set as 126 μs, the flow rate of the enzyme solution was 22 cm/s, and it took about 0.1 s for the enzyme solution to get to the temperature checkpoint from the chamber and about 0.7 s to get to the cooling coil from the temperature checkpoint. The temperatures of enzyme solution before (T_0) and after (T_7) PEF or heat treatment were measured by mercury thermometer. A micro gear pump (Model 020-000-010, Micropump, Inc., Vancouver, Wash., U.S.A.) 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), and t is the total treatment time (μs) defined by

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

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

Heat treatment

Heat treatment was conducted with the same set of coils with PEF chambers, which was used in PEF treatment. However, put the first 2 coils into 1 water bath for heating and the last coil into a 0 °C ice-water bath for cooling (Figure 3). The enzyme solution was pumped through the heating and cooling system by the same fluid-handling system that was used in PEF treatment. Similar temperature profile was obtained by adjusting the temperature of the water bath and the flow speed of the enzyme solution. Samples were collected from the outlet of the last cooling coil and immediately put into a 0 °C ice-water bath and quickly shaken for further cooling. Temperatures at checkpoints were monitored during heat treatment by a dual-channel digital thermocouple reader (Fisher Scientific) as in PEF treatment. The distance between the temperature checkpoint and the surface of hot water in the bath was about 15 cm. When the flow rate was set as 22 cm/s, which was equal to the flow rate in a PEF treatment with a total treatment time

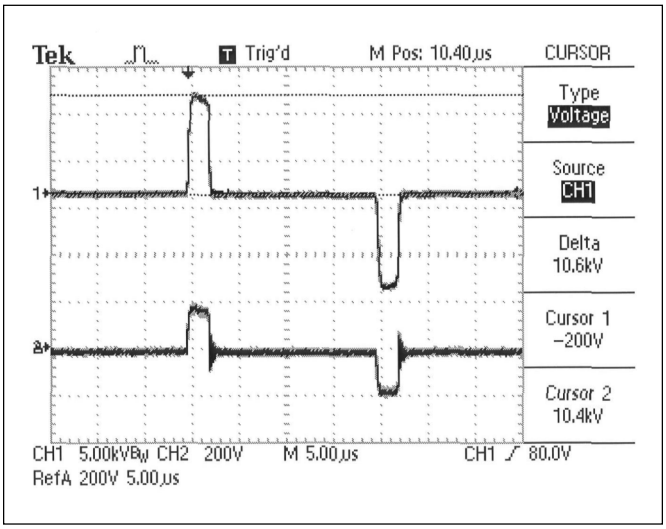

Figure 2—Bipolar square-wave pulse pair. Trace 1 is the measured voltage with 5 kV per division. Trace 2 is the measured current with 20 A per division. Time scale is 5 μs per division.

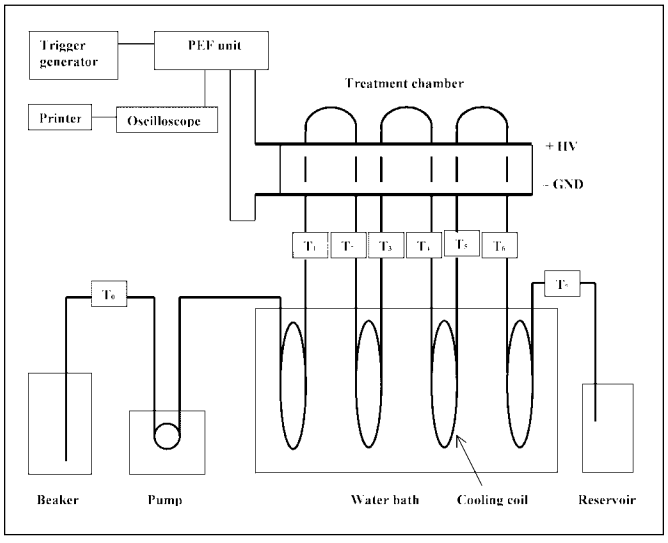

Figure 1—Diagram of the pulsed electric field (PEF) bench-scale processing unit (OSU-4A). Temperatures of inlet (T_1, T_3, and T_5) and outlet (T_2, T_4, and T_6) of each pair of chambers were measured by thermocouples. T_0 and T_7 were measured by mercury thermometers.

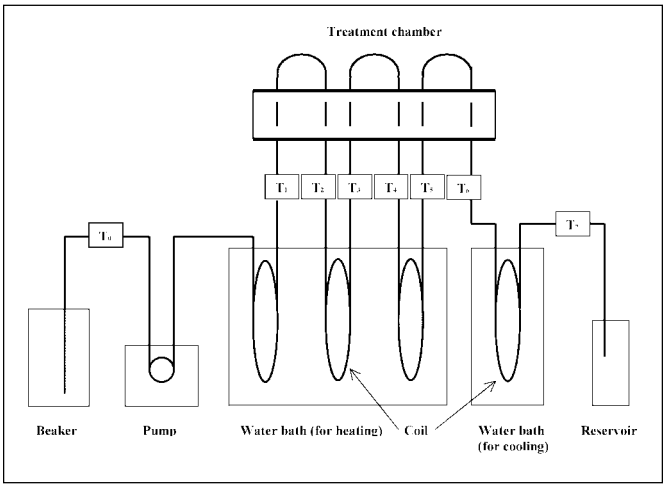

Figure 3—Diagram of the heat treatment unit. Temperatures of inlet (T_1, T_3, and T_5) and outlet (T_2, T_4, and T_6) of each pair of chambers were measured by thermocouples. T_0 and T_7 were measured by mercury thermometers.

Table 2—Assay method and apparatus for each enzyme

			Major parameters		
Enzyme	Assay method	Major apparatus	Reaction condition	Wavelength and light path	Reference
Pepsin	Spectrophotometric stop rate determination	Spectronic Genesys 5 spectrometer (Milton Roy, Rochester, N.Y., U.S.A.)	38 °C, 10 min	280 nm, 1.0 cm	Anson 1938
Lysozyme	Continuous turbidmetric rate determination	UV-2401 PC spectrophotometer (Shimadzu Co., Kyoto, Japan)	25 °C, pH 6.2, 5 min	450 nm, 1.0 cm	Shugar 1952
Peroxidase	Continuous spectrophotometric rate determination	UV-2401 PC spectrophotometer	20 °C pH 6.0 5 min	450 nm 1.0 cm	Chance and Maehly 1955
Polyphenol oxidase	Continuous spectrophotometric rate	UV-2401 PC spectrophotometer determination	25 °C, pH 6.5, 8 min	280 nm, 1.0 cm	Duckworth and Coleman 1970
Chymotrypsin	Continuous spectrophotometric rate determination	UV-2401 PC spectrophotometer	25 °C, pH 7.8, 5 min	256 nm, 1.0 cm	Wirnt 1974

of 126 μs, it took about 0.7 s for the enzyme solution to get into or out of the heating coils from the corresponding temperature checkpoints.

Measurements of enzyme activity

Standard enzymatic assay procedures (Sigma Co.) were followed to prepare the reagents and determine the enzyme activity of untreated (control), PEF-treated, or heat-treated enzyme solutions. The assays, major apparatus, and parameters are shown in Table 2.

Relative residual activity

The relative residual activity (RRA) of enzymes was expressed as percentage of the activity of the treated enzyme solution to the control. For PEF treatment, the control samples passed through the whole system at the same water-bath temperature as it was during PEF treatment, but without turning on PEF. For heat treatment, the control passed through the system when the temperatures of the both water baths were set at 0 °C. Before activity assay, all samples were kept in 0 °C ice-water bath.

Calculation of adiabatic temperature increase and adiabatic temperature increase constant

The calculation of adiabatic temperature increase was calculated using the following equation:

$$\Delta T = (V I \tau f t_r) / (C_p v \rho) \quad (4)$$

where ΔT is the adiabatic temperature increase (°C); V is the peak pulse voltage across a PEF chamber; I is the peak current passing through a PEF chamber (amps); τ is the pulse width (s); f is the pulse repetition rate (pps); t_r is the resident time of sample within a PEF chamber; C_p is the specific heat of sample (J/g·°C); v is the volume of the PEF chamber (cm^3); and ρ is the density of the sample (g/cm^3).

The current, I, was determined by

$$I = V / R = V\sigma A / (dF_R) \quad (5)$$

where d is the gap distance between the 2 electrodes in a PEF chamber (m); σ is the electric conductivity of the sample (S/m); and A is the cross sectional area of the treatment zone (m^2).

In a cofield flow PEF chamber, the electrodes are tubular sections rather than flat-end surfaces of the cylindrical sample. Therefore, the equivalent load resistance, R, carries a correction factor F_R, which was determined experimentally as the ratio of calculated current, assuming flat-end cylindrical sample and measured current.

Statistical analyses

All experiments were duplicated at lease twice, and the assay for enzyme activity of each sample was replicated at the same time. The data presented are the means of each experiment. Analysis of variance was performed to determine variations among treatments.

Results and Discussion

Inactivation of selected enzymes by PEF treatment

PEF treatments for each enzyme were carried out at same conditions (inlet temperature 4 °C, ice-water bath for cooling between each pair of chambers, 2 μs pulse width, 800 pps pulse repetition rate, 126 μs total treatment time), except the media and electric field strength. Figure 4 illustrates the effect of PEF treatment on the activity of pepsin, lysozyme, peroxidase, polyphenol oxidase, and chymotrypsin. The sensitivities of enzymes to PEF are different from enzyme to enzyme. The inactivation efficiency of PEF on enzymes increases with the increase of electric field strength when the total treatment time and other parameters were maintained same. Pepsin exhibited an activity reduction of 35.1% at 32.9 kV/cm, 51.7% at 37.0 kV/cm, and 83.8% at 41.8 kV/cm. Polyphenol oxidase showed an activity reduction of 16.1% at 28.8 kV/cm and 38.2% at 33.6 kV/cm. Peroxidase showed an activity reduction of 8.4% at 31.5 kV/cm and 18.1% at 34.9 kV/cm. Only 4% of chymotrypsin activity was inactivated after PEF treatment at 34.2 kV/cm for 126 μs. Lysozyme was not affected in activity by PEF treatment under the tested conditions. Pepsin and polyphenol oxidase are more sensitive to PEF treatment than peroxidase, chymotrypsin, and pepsin.

A slight increase of activity of pepsin after PEF treatment at 10 to 20 kV for 126 μs was observed. Ho and others (1997) argued that PEF does not inactivate pepsin but enhances its activity. With the increase of electric field strength, the residual activity was increased by 240% of its original activity at 35 kV/cm, and no inactivation was observed in the range of 0 to 70 kV/cm for 60 μs. Figure 4 indicates that when the dosage of PEF treatment was less than 20 kV/cm for 126 μs there was no detectable inactivation. The reduction of activity rapidly increased with the increase of electric field strength higher than 20 kV/cm. No pepsin activity was detected after PEF treatment was achieved at 35 kV/cm for 350 μs.

Figure 4 revealed that PEF treatment at 0 to 38 kV/cm for 126 μs has no detectable inactivation effect on lysozyme activity. Ho and others (1997) reported that with the increase of electric field strength, the residual activity of lysozyme decreased first (0 to 15 kV/cm, 60 μs) then increased (15 to 45 kV/cm, 60 μs), and decreased again after reached the highest point after PEF treatment at 45 kV/cm for 60 μs.

Both polyphenol oxidase and peroxidase are of concerns in fruit and vegetable preservation. Inactivation of polyphenol oxidase and peroxidase is required in the early step of processing to avoid the damage of the flavor, color, and nutrient of the product. Polyphenol oxidase and peroxidase were not fully inactivated under the normal PEF dosage required by inactivating microorganisms such as 35 kV/cm for 59 μs for orange juice (Ayhan and others 2001), 34 kV/cm for 166 μs for apple juice and cider (Evrendilek and others 2000), and 40 kV/cm for 57 μs for tomato juice (Min and others 2003b). Higher inactivation of polyphenol oxidase by PEF treatment with higher dosages were reported, for instance, 97% in apple extract at 24.6 kV/cm for 6000 μs, 72% in pear extract at 22.3 kV/cm for 6000 μs (Giner and others 2001), and 70% in peach extract at 24.30 kV/cm for 5000 μs (Giner and others 2002).

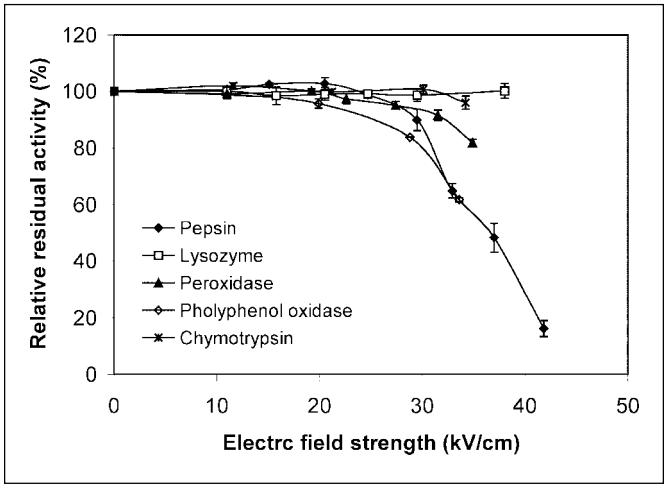

Figure 4—Inactivation of 5 enzymes by pulsed electric field (PEF). The pulse width and total treatment time were 2 μs and 126 μs, respectively. The media and pH and electrical conductivity for different enzymes are as follows. Pepsin: 7 m*M* HCl; pH 2.1, 0.352 S/m; the adiabatic temperature increase was 42 °C per pair of chambers at 41.1 kV/cm for 126 μs. Lysozyme: 25 m*M* potassium phosphate buffer; pH 6.5; 0.345 S/m; the adiabatic temperature increase was 34 °C per pair of chambers at 38.2 kV/cm for 126 μs. Peroxidase: 25 m*M* potassium phosphate buffer; pH 6.0; 0.341 S/m; the adiabatic temperature increase was 25 °C per pair of chambers at 34.2 kV/cm for 126 μs. Polyphenol oxidase: 25 m*M* potassium phosphate buffer; pH 6.4; 0.342 S/m; the adiabatic temperature increase was 22 °C per pair of chambers at 32.7 kV/cm for 126 μs. Chymotrypsin: 1 m*M* HCl + NaCl; pH 2.9; 0.343 S/m; the adiabatic temperature increase was 26 °C per pair of chambers at 34 kV/cm for 126 μs. The sample exposed to the adiabatic temperature before cooling for 3 time periods of 0.12 s.

Temperature increase induced during PEF treatment was observed. The correction factor calculated with Eq. 5 was between 1.15 and 1.35, depending on the electric conductivity of the sample and the voltage applied because the cooling did not completely cool the sample back to the inlet temperature. The maximum adiabatic temperature increase was 42 °C, 34 °C, 25 °C, 22 °C, and 26 °C per pair of chambers at the maximum dosages applied in the studies as illustrated in Figure 4. The traveling distance from the exit of the chamber to cooling ice bath was 10 cm and the sample exposed to the enhanced temperature for as short as 0.12 s. With inlet temperature at 4 °C, the maximum temperature that the samples exposed to was 46 °C, 38 °C, 29 °C, 26 °C, and 30 °C for pepsin, lysozyme, peroxidase, polyphenol oxidase, and chymotrypsin, respectively. Exposing the enzyme solutions to these temperature for 0.12 s can contribute minimally (less than 5%) to the observed inactivation effect on enzyme activity.

In general, the larger an enzyme and the more complex its structure, the more susceptible it is to high temperature. However, the data shown in Figure 4 suggest that the sensitivity of the tested enzymes to PEF has no relationship with their thermostability and size. Peroxidase is the most thermostabe enzyme among all of the tested enzymes, but it is not the most PEF resistant. Pepsin, the more sensitive enzyme to PEF among the tested enzymes, has a molecular weight of 35500, the size of which is between lyzozyme (MW 14300) and peroxidase (MW 44000). The structure of polyphenol oxidase (a tetramer with a molecular weight of 128000) is much larger and more complex than that of pepsin (a monomer). However, the sensitivity of these 2 enzymes to PEF is similar. Therefore, the mechanism involved in inactivation of enzymes by PEF may be different from that by heat.

Effect of heat produced by PEF treatment on the inactivation of enzymes

Enzymes are heat-labile substances. PEF treatment will generate minimal amount heat, depending on the selected dosage and media used. The induced heat will in turn cause increase in temperature that, when high enough, may cause thermal inactivation of enzymes. It remains an issue (Van Loey and others 2002) whether PEF or induced heat or both are responsible to the inactivation of enzymes. To investigate the thermal contribution during PEF treatment, the same coil unit with the treatment chambers used in PEF inactivation study was used for thermal inactivation tests. The coils before the 3rd pair of chambers were immersed in the hot-water bath. The coils after the 3rd pair of chambers were immersed in the ice-water bath (Figure 3) to cool the enzyme solution immediately after treatment. The highest temperature in the heat treatment was kept slightly higher than the highest temperature obtained in PEF treatment. Because there were cooling coils between 2 pairs of PEF chambers, the actual heat treatment dosage is more severe in the heat treatment setup than that in the PEF setup. The temperature profile that the pepsin and polyphenol oxidase experienced and enzyme activity reduction after PEF or heat treatment are shown in Figure 5. When the highest temperature was about 50 °C, the thermal inactivation of pepsin was less than 5%, whereas the inactivation by PEF achieved 54%. The effect of PEF on the inactivation of pepsin was significant ($P < 0.05$). The same conclusion could be extracted from the results of polyphenol oxidase tests. When the highest temperature is 5 °C lower in the PEF test than that in the thermal test, the enzyme activity reduction (36.8%) in the PEF test was 19.4% higher than that (17.4%) in the

thermal test. Pepsin is more sensitive to PEF treatment than polyphenol oxidase.

For the experiments shown in Figure 5, the temperature profile of each enzyme experienced during PEF treatment was similar at the same electric field strength because of the similar electrical conductivity of each enzyme. In general, when an electric field of 33.5 to 35 kV/cm and a total PEF treatment of 126 μs were applied, the highest measured temperature is in the range of 42 °C to 43 °C, lower than that shown in Figure 5. Although heat produced during PEF treatment contributed to the inactivation of enzyme, most of the reduction of activity shown in Figure 4 was achieved by PEF.

Effect of electric field strength on the inactivation of pepsin

To evaluate the effect of heat produced during PEF treatment on the inactivation of enzymes, further experiments were conducted with the change of temperature in each pair of PEF chambers maintained constant; the results are illustrated in Figure 6. Temperature increase of enzyme solution with the same electrical conductivity is an indicator of the PEF dosage received by enzyme solution. To maintain the temperature increase in each pair of chambers constant, the treatment time was changed with the change of electric field strength. It was achieved by adjusting the flow rate of enzyme solution. The data in Figure 6 suggest that RRA of pepsin decreases with the increase of electric field strength with the same temperature increase. When the temperature increase in each pair of chambers was 23 °C and 28 °C, the activity reduction of pepsin was increased from 18.8% and 36.4% at 17.8 kV/cm to 34.6% and 60.1% at 32.9 kV/cm, respectively. Higher electric field strength was more effective in inactivating pepsin.

The influence of electrical conductivity and pH of enzyme solution

The pH and ion strength of the system has critical influence on enzymes. Buffers instead of real food were used in this study for simplicity and repeatability. For the pepsin study, HCl solution was selected as the medium for pH 2.0. The electrical conductivity of 7 m*M* HCl with a pH of 2.1 is higher than 0.3 S/m. For the study of polyphenol oxidase, 10 m*M*, pH 6.5 potassium phosphate was chosen as the basic medium, and the electrical conductivity was adjusted by adding NaCl. Figure 7 shows the influence of electrical conductivity on inactivation of pepsin, polyphenol oxidase, and chymotrysin by PEF. Electrical conductivity has a significant effect on the inactivation of pepsin and polyphenol oxidase but not chymotrypsin (Figure 7). In fact, PEF had no significant influence on the activity of chymotrypsin under the tested condition. The higher the electrical conductivity of the medium, the more effective the PEF inactivation of pepsin and polyphenol oxidase. Pepsin RRA decreases more rapidly in a medium with a conductivity of 0.385 S/m than that with a conductivity of 0.307 S/m when electric field strength increased higher than 20 kV/cm. The same trend was also observed for polyphenol oxidase.

PEF treatment with higher electrical conductivity media will induce more heat and higher temperature increase at the same electric field strength for same treatment time. The adiabatic temperature increases in the enzyme solutions were calculated (data not shown) and matched well with the measured temperatures. The difference in enzyme inactivation effect by PEF between the samples treated in media with different electric conductivity is illustrated in Figure 7a and 7b. The difference may contain some contribution of the heat. However, the difference is caused mainly by PEF because the curves begin departure at 20 kV/cm, at which the maximum adiabatic temperature increase (among all the conditions tested in this study) per pair of chamber was less than 15 °C. When the electric field strength was lower than 30 kV/cm, the highest temperature the enzymes experienced was lower than 40 °C, which does not cause detectable activity loss of enzyme activity. Because of differences in the electrical conductivity in different food products, the sensitivity of certain enzymes may vary in different system.

Most charged residues are distributed on the surface of a protein, and less than 5% are buried inside the globular molecule. Structural stability and functional properties of proteins are because of a delicate balance of a number of interactions, mainly noncovalent in nature. Among them, electrostatic interactions are critical factors (Koumanov and others 2001). The electrostatic interactions are functions of pH, temperature, ion strength, and electric field. Higher electrical conductivity means more free ions in the solution. High concentration of free ions may assist suppress or disrupt the electrostatic interactions under pulsed electric field.

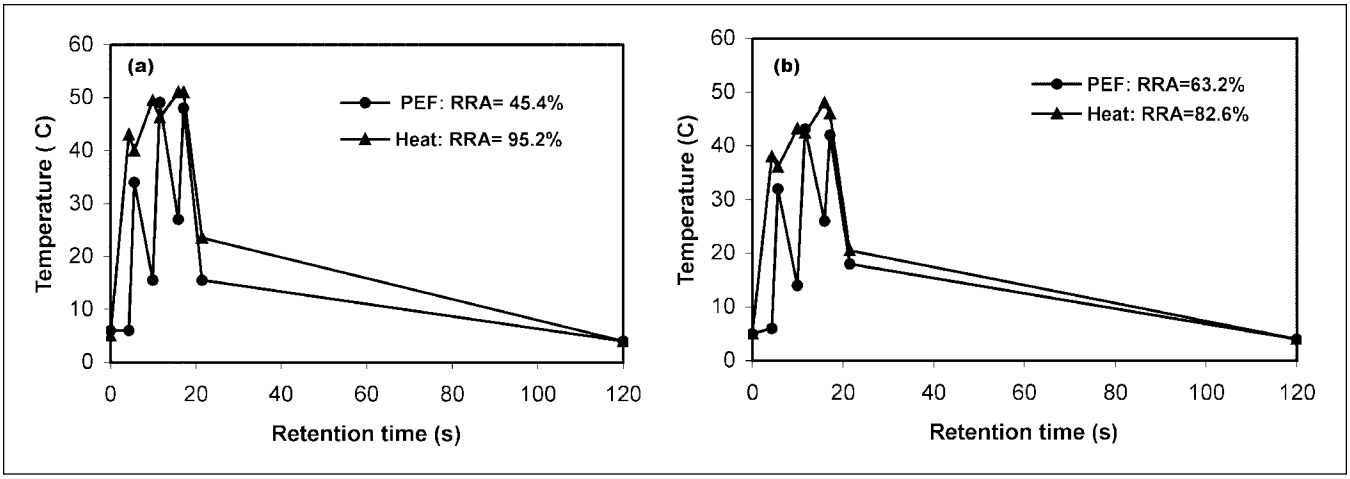

Figure 5—Profile of the temperature change of pepsin (a) and polyphenol oxidase (b) solution during heat or pulsed electric field (PEF) treatment. PEF refers to a PEF inactivation test of 39.0 kV/cm for 126 μs for pepsin and 32.8 kV/cm for 126 μs (a) for polyphenol oxidase (b). Heat refers to a thermal inactivation test. RRA represents the relative residual activity.

Under the conditions described in Figure 7, the maximum adiabatic temperature increase per pair of chambers was 30 °C at 37.2 kV/cm with 0.307 S/m buffer and 38 °C at 37.8 kV/cm with 0.385 S/m medium, 13 °C at 41.1 kV/cm with 0.112 S/m buffer, 26 °C at 34.2 kV/cm with 0.342 S/m, 13 °C at 44 kV/cm with 0.099 S/m, and 25 °C at 32.4 kV/cm with 0.343 S/m medium for pepsin, polyphenol oxidase, and chymotrypsin, respectively. With an inlet temperature of 4 °C, the maximum temperature for tests illustrated in Figure 7 was 42 °C. Exposing the 3 enzyme solutions at 40 °C for 0.36 s (3 × 0.12 s) causes no detectable loss of enzyme activity.

Table 3 shows the influence of both of electrical conductivity and pH on the inactivation of pepsin by PEF. The data show that both pH and conductivity are critical factors. The RRA of pepsin decreased from 74.1% to 44.1% whereas the pH increased from 2.1 to 6.4 in almost the same conductivity and PEF treatment. In an optimum pH of 2.0, pepsin had highest stability against PEF. The RRA of pepsin decreased from 90.2% to 44.1% whereas the conductivity increased from 0.132 to 0.301 S/m in the same pH (pH 6.4) and PEF treatment. Different media with different electrical conductivity were used as the medium for a certain enzyme by different research groups. Some conflicting results reported by different research groups might be caused by the difference in electrical conductivity of the media. Calculated adiabatic temperature increase induced by PEF was summarized in Table 3. However, because the exposing time for samples at the enhanced temperature was only 0.12 s, we attributed little of the observed inactivation effects to the induced temperature increase.

Table 3—The influence of pH and conductivity on the inactivation of pepsin by pulsed electric field (PEF) treatment[a]

Conditions					
Media	pH	Conductivity (S/m)	Electric fields strength (kV/cm)	Relative residual activity (%)	ΔT[b] (°C)
7 m*M* HCl	2.1	0.302	34.2	74.1 ± 0.6	24.4
	2.1	0.302	38.4	48.1 ± 1.3	31.2
1 m*M* HCl + NaCl	2.9	0.301	34.2	61.7 ± 1.0	23.0
10 m*M* KPB[c]	6.4	0.132	37.7	90.2 ± 1.4	14.2
	6.4	0.132	41.8	82.2 ± 0.4	15.8
10 m*M* KPB + NaCl	6.4	0.301	34.2	44.1 ± 1.9	24.2

[a]Sample exposing time to the enhanced temperature was 0.12 s.
[b]ΔT refers to the calculated adiabatic temperature increase per pair of chambers (°C).
[c]KPB refers to potassium phosphate buffer.

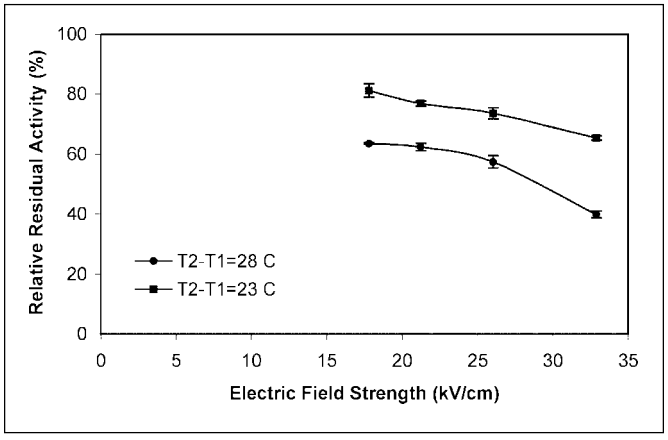

Figure 6—Effect of electric field strength on the inactivation of pepsin

Effect of temperature on the efficiency of PEF on inactivating enzymes

Potassium phosphate buffer (10 m*M*, pH 6.4) was selected for the study of effect of temperature on the efficiency of PEF on inac-

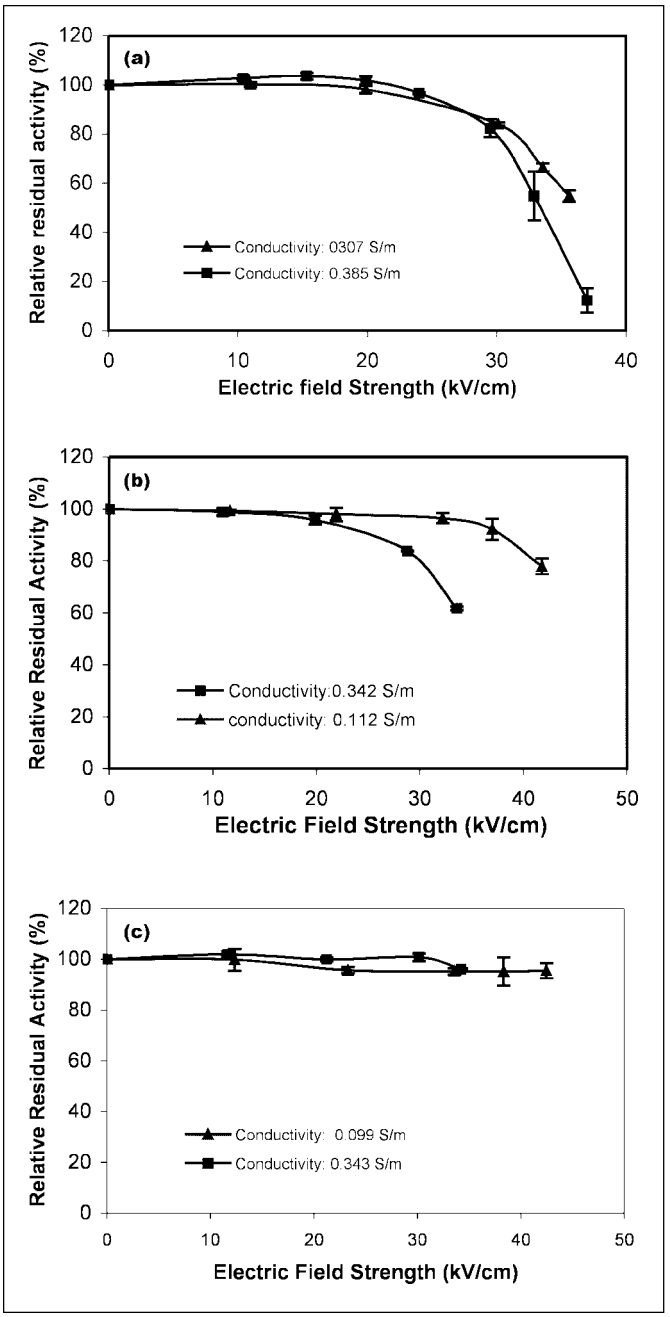

Figure 7—The influence of electrical conductivity on the inactivation of enzymes by pulsed electric field (PEF). The pulse width and total treatment time were 2 μs and 126 μs, respectively. (a) pepsin; (b) polyphenol oxidase; (c) chymotrypsin by PEF.

Table 4—Effect of temperature on the inactivation of pepsin and polyphenol oxidase by pulsed electric field (PEF)[a]

Water bath temperature (°C)	Highest temperature[b] (°C)	Relative residual activity (%)	
		Pepsin	Polyphenol oxidase
0	25.5	85.2 (±0.8)	97.1 (±1.4)
5	30.3	81.8 (±0.3)	
15	38.2	69.2 (±1.7)	
20	41.3	68.5 (±1.1)	91.3 (±2.2)

[a]The electric field strength and total treatment time were 37.7 kV/cm and 126 μs, respectively.
[b]Highest temperature refers to the highest temperature (T_6) experienced by enzyme during PEF treatment.

tivating pepsin. A relatively low electrical conductivity of 0.128 S/m was chosen to minimize the effect of heat. The temperature profile during PEF treatment was controlled by adjusting the temperature of water bath.

Table 4 shows the effect of temperature on the efficiency of PEF on inactivating pepsin and polyphenol oxidase, respectively. The effect of heat produced during PEF treatment in all experiment shown in Table 4 shall be ignored because of the relatively low temperature. The data indicate that PEF is more effective in inactivating pepsin and polyphenol oxidase in higher temperature. It is expected because higher temperature results in higher mobility of charged groups and dipoles of enzyme protein under the electric field.

Conclusions

The sensitivity of enzymes to PEF treatment varies from enzyme to enzyme. The sequence of sensitivity to PEF of the 5 tested enzymes at their optimum pH from high to low is pepsin, polyphenol oxidase, peroxidase, chymotrypsin, and lysozyme. Lysozyme activity was not affected by PEF below 38 kV/cm. Both PEF and the induced heat by PEF treatment contribute to the inactivation of enzymes. The contribution of PEF and the induced heat to the observed enzyme inactivation depends on the properties of enzymes and the test conditions, including the efficiency of the cooling system. The inactivation effect of PEF on enzymes was affected by electric field strength, electrical conductivity, and pH of the media, especially the electrical conductivity. Some conflicting results reported by different research groups may be caused by the differences in electrical conductivity of media.

Acknowledgments

Funding for this project was from The US Army Natick Soldier System Center, Ohio Agricultural Research and Development Center, and the China Scholarship Council.

References

Anson ML. 1938. The estimation of pepsin, typsin, papain, and cathepsin with hemoglobin. J Gen Physiol 22:79–89.
Ayhan Z, Yeom HW, Zhang QH, Min DB. 2001. Flavor, color, and vitamin C retention of pulsed electric field processed orange juice in different packaging materials. J Agric Food Chem 49(2):669–74.
Ayhan Z, Zhang QH, Min DB. 2002. Effects of pulsed electric field processing and storage on the quality and stability of single-strength orange juice. J Food Protect 65(10):1623–7.
Barbosa-Canovas GV, Pierson MD, Zhang QH, Schaffner DW. 2000. Pulsed electric fields. J Food Sci (Spec. Suppl.: Kinetics of Microbial Inactivation for Alternative Food Processing Technologies):65–81.
Bendicho S, Barbosa-Canovas GV, Martin O. 2003. Reduction of protease activity in milk by continuous flow high-intensity pulsed electric field treatments. J Dairy Sci 86(3):697–703.
Brooks DW, Dawson CR. 1966. Aspects of tyrosinase chemistry. In: Peisach J, Aisen P, Blumberg WE, editors. The biochemistry of copper. New York: Academic Press. p 343–57.
Castro AJ, Swanson BG, Barbosa-Canovas GV, Zhang QH. 2001. Pulsed electric field modification of milk alkaline phosphatase activity. In: Barbosa-Canovas GV, Zhang QH, Tabilo-Munizaga G, editors. Pulsed electric fields in food processing. Lancaster, Pa.: Technomic Publishing. p 65–82.
Chance B, Maehly AC. 1955. preparation and assays of enzymes. In: Colowick SP, Kaplan NO. Methods Enzymol II. New York, N.Y.: Academic Press Inc. p 773–5.
Duckworth HW, Coleman JE. 1970. Physicochemical and kinetic properties of mushroom tyrosinase. J Biol Chem 245:1613–25.
Evrendilek GA, Jin ZT, Ruhlman KT, Qiu X. 2000. Microbial safety and shelf-life of apple juice and cider processed by bench and pilot scale PEF systems. Innov Food Sci Emerg Technol 1:77–86.
Giner J, Gimeno V, Barbosa-Canovas GV, Martin O. 2001. Effects of pulsed electric field processing on apple and pear polyphenoloxidases. Food Sci Tech Int 7(4):339–45.
Giner J, Ortega M, Mesegue M, Gimeno V, Barbosa-Canovas GV, Martin O. 2002. Inactivation of peach polyphenoloxidase by exposure to pulsed electric fields. J Food Sci 67(4):1467–72.
Grahl T, Märkl H. 1996. Killing of microorganisms by pulsed electric fields. Appl Microbiol Biotech 45(1/2):148–57.
Ho SY, Mittal GS, Cross JD. 1997. Effect of high field electric pulses on the activity of selected enzymes. J Food Eng 31:69–84.
Jolley RLJ, Nelson RM, Robb DA. 1969a. The multiple forms of mushroom tyrosinase. J Bio Chem 244:3251–7.
Jolley RLJ, Robb DA, Mason HS. 1969b. The multiple forms of mushroom tyrosinase. Association-dissociation phenomena. J Bio Chem 244:1593–9.
Knorr D. 1999. Novel approaches in food-processing technology: new technologies for preserving foods and modifying function. Curr Opin Biotech 10(5):485–91.
Koumanov A, Ladenstein R, Karshikoff A. 2001. Electrostatic interactions in proteins: contribution to structure-function relationships and stability. Recent Res Dev Protein Eng 1(1):123–48.
Li SQ, Zhang QH, Lee YZ, Pham TV. 2003. Effects of pulsed electric fields and heat treatment on the stability of Bovine Immunoglobulin G (IgG) in enriched soymilk. J Food Sci 68(4):1201–7.
Mertens B, Knorr D. 1992. Developments of nonthermal processes for food preservation. Food Tech 46(5):125–33.
Min S, Jin ZT, Min SK, Yeom H, Zhang QH. 2003a. Commercial-scale pulsed electric field processing of orange juice. J Food Sci 68:1265–71.
Min S, Jin ZT, Zhang QH. 2003b. Commercial scale pulsed electric field processing of tomato juice. J Agric Food Chem 51:3338–44.
Shugar D. 1952. The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. Biochem Biophys Acta 8:302–9.
Van Loey A, Verachtert B, Hendrichx M. 2002. Effects of high electric field pulses on enzymes. Trends Food Sci Tech 12:94–102.
Vega-Mercado H, Powers JR, Barbosa-Canovas GV, Swanson BG. 1995a. Plasmin inactivation with pulsed electric fields. J Food Sci 60:1143–6.
Vega-Mercado H, Powers JR, Barbosa-Canovas GV, Swanson BG, Luedecke L. 1995b. Inactivation of a protease from *Pseudomonas fluorescens* M3/6 using high voltage pulsed electric fields. In: Book of abstracts. Proceedings IFT Annual Meeting; June 3-7; Annaheim, Calif. Chicago, Ill.: IFT. p 267.
Vega-Mercado H, Powers JR, Martin-Belloso O, Luedecke L, Barbosa-Canovas GV, Swanson BG. 2001. Change 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: Barbosa-Canovas GV, Zhang QH, Tabilo-Munizaga G, editors. Pulsed electric fields in food processing. Lancaster, Pa.: Technomic Publishing. p 105–20.
Wirnt R. 1974. In: Bergmeyer, editor. Methods of enzymatic analysis. 2nd ed. Volume II. New York: Academic Press. p 1009–12
Whitaker JR. 1994. Principles of enzymology for the food science. 2nd ed. New York: Marcel Dekker. p 302.
Yeom HW, Streaker CB, Zhang QH, Min DB. 2000a. Effects of pulsed electric fields on the activities of microorganisms and pectin methyl esterase in orange juice. J Food Sci 65(8):1359–63.
Yeom HW, Streaker CB, Zhang QH, Min DB. 2000b. Effects of pulsed electric fields on the quality of orange juice and comparison with heat pasteurization. J Agric Food Chem 48(10):4597–605.
Yeom HW, Zhang QH, Dunne CP. 1999. Inactivation of papain by pulsed electric fields in a continuous system. Food Chem 67:53–9.
Zhang QH, Qiu X, Sharma SK. 2002. PEF process improves safety and quality of orange juice. In: Yang XH, Tang J, editors. Advances in agricultural science and technology. Vol. 1. Advances in bioprocess engineering. River Ridge, N.J.: World Scientific. p 45–67.