Critical Electric Field Strengths of Onion Tissues Treated by Pulsed Electric Fields

Suvaluk Asavasanti, Seda Ersus, William Ristenpart, Pieter Stroeve, and Diane M. Barrett

Abstract: The impact of pulsed electric fields (PEF) on cellular integrity and texture of Ranchero and Sabroso onions (*Allium cepa* L.) was investigated. Electrical properties, ion leakage rate, texture, and amount of enzymatically formed pyruvate were measured before and after PEF treatment for a range of applied field strengths and number of pulses. Critical electric field strengths or thresholds (E_c) necessary to initiate membrane rupture were different because dissimilar properties were measured. Measurement of electrical characteristics was the most sensitive method and was used to detect the early stage of plasma membrane breakdown, while pyruvate formation by the enzyme alliinase was used to identify tonoplast membrane breakdown. Our results for 100-μs pulses indicate that breakdown of the plasma membrane occurs above $E_c = 67$ V/cm for 10 pulses, but breakdown of the tonoplast membrane is above either $E_c = 200$ V/cm for 10 pulses or 133 V/cm for 100 pulses. This disparity in field strength suggests there may be 2 critical electrical field strengths: a lower field strength for plasma membrane breakdown and a higher field strength for tonoplast membrane breakdown. Both critical electric field strengths depended on the number of pulses applied. Application of a single pulse at an electric field up to 333 V/cm had no observable effect on any measured properties, while significant differences were observed for $n \geq 10$. The minimum electric field strength required to cause a measurable property change decreased with the number of pulses. The results also suggest that PEF treatment may be more efficient if a higher electric field strength is applied for a fewer pulses.

Keywords: critical electrical field strength, electrical properties, pulsed electric fields, pyruvate formation, tissue integrity

Introduction

Consumer interests in healthier and fresher foods have driven much of the interest in nonthermal or minimal processes that are safe but at the same time capable of imparting fresh-like characteristics to processed food. Pulsed electric field (PEF) treatments are considered a promising minimal processing alternative and this technology has recently received considerable attention in food related applications (Lund 2008). PEF treatment is conveyed by application of DC voltages for very short periods of time, often on the order of micro second, through a food material placed between 2 electrodes. The electrical discharges are applied in the form of a number of short pulses. A typical set-up consists of a pulse generator, treatment chamber, data acquisition, control system, and materials-handling equipment (San Martin and others 2003).

The PEF effects can vary depending on treatment conditions, including peak voltage delivered to the treatment chamber, power of the system, pulse shape, duration of each pulse (t_i), and pulse number (n). The gap between the electrodes and the voltage delivered to the treatment chamber determine the strength of the electric field (E), which is often reported in units of V/cm. In general, field strengths of $E < 100$ V/cm are considered to be low-intensity electric fields, while E in the range of 0.1 to 1 kV/cm are considered to be moderate electric fields, and $E >$ 5 kV/cm are considered to be high-intensity electric fields (Fincan and Dejmek 2002; Lebovka and others 2002; Rastogi 2003; Loeffler 2006). In previous studies, the gap between electrodes varied from approximately 0.5 cm, or smaller for pilot scale PEF systems, up to 1 cm in commercial applications for liquid food or juice products (San Martin and others 2003; Picart and Cheftel 2003). In solid foods, such as potato, beet, and carrot, the size of electrode gaps used are in range of 0.5 to 2 cm (Angersbach and others 2000; Bazhal and others 2001; Lebovka and others 2001). The most generally applied pulse geometries are exponential decay and square waveform. Knorr and others (1994a) indicated that the square waveform is more desirable than the exponential decay waveform to minimize the absorption energy of treated systems, which will be transformed into heat.

There is insufficient information regarding the effect of pulse width (or pulse duration) on the PEF-induced disintegration of tissue at the fixed total treatment time (Vorobiev and Lebovka 2008). The effect of pulse width was mainly explored on microbial inactivation and it seems to vary depending on electric field strength; however, the results were still inconclusive. Some researchers observed no pulse width influence on microbial inactivation (Raso and others 2000; Mañas and others 2001; Sampedro and other 2007). On the other hand, Wouters and others (1999) reported significant effect of pulse width ($t_i = 2$, 3, and 3.9 μs) on inactivation of *L. innocua* NCTC 11289 at 2 electric field strengths ($E = 2.8$ and 3.6 V/μm) and the relationship between energy input and pulse width during inactivation depended on the electric field strength. De Vito and others (2008) reported that the efficiency of apple tissue disintegration by PEF increased with

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increasing pulse width. This finding was well correlated with the theory of membrane charging time prediction for large cell in soft tissue, proposed by Kotnik and others 1998. Depending on the cell dimensions, larger cells, such as plant cell, require more time to charge up the membrane than the microbial cells. Pulse width is not very critical for microbial inactivation since the charging time is commonly less than 1 μs and higher PEF efficiency could be expected when pulse width is higher than the membrane charge time (De Vito and others 2008). The electrical properties of the food to be processed, primarily its electrical conductivity, are the most important determinants of field strength. Electrical resistivity is the inverse of the electrical conductivity. Electrical conductivity indicates the ability of electrical current to pass thorough a material in Siemens per centimeter (Dunn 2001).

Many studies have found that PEF causes permeability changes in cell membranes of biological materials (Knorr and others 1994a, 1994b; Zimmermann 1986; Weaver and Chizmadzhev 1996; and references therein). Externally applied electric fields affect cellular systems in many ways, ranging from low-intensity field effects associated with signaling, wound healing, cell growth, and transport to relatively high-intensity pulsed fields that can cause changes in the integrity of the cell membrane (Weaver and Chizmadzhev 1996; Angersbach and others 2000; Tekle and others 2005). An electrical field can cause a dramatic increase in membrane permeability and a decrease in the electrical resistance, if the electrical field is greater than the critical threshold value for the membrane. Irreversible membrane rupture due to the appearance of large pores in cell membranes can take place. This phenomenon is often called membrane breakdown, or membrane permeabilization, or electroporation of the membrane (Zimmermann 1986; Lebovka and others 2004).

Pulsed electric fields (PEF) have recently been used as a novel alternative for food dehydration by removal of water from within the cell through membrane breakdown. PEF have also been applied for microbial inactivation and juice extraction (Qin and others 1998; Bazhal and Vorobiev 2000; Bajgai and Hashinaga 2001; Bazhal and others 2001; Taiwo and others 2002; Picart and Cheftel 2003). Pulsed electric field effects on cell membranes may be useful in applications such as juice extraction from plant tissues, but they may also cause undesirable changes in texture, color, flavor, aroma, and nutrient content of fruits and vegetables (Vazquez-Tello and others 1990; Diaz-Maroto and others 2004; Gonzalez 2009). Because PEF treatments disrupt cell membranes, the cellular turgor component of texture is removed and there is a significant influence on the viscoelastic properties of plant tissues (Fincan and Dejmek 2003; Lebovka and others 2003).

Previous studies have found that plant tissue disruption under PEF treatments can be achieved at room temperature using moderate electric fields of 0.5 to 5 kV/cm within 10^{-4} to 10^{-2} s, whereas for breakdown of microbial membranes, field strengths of 15 kV/cm and higher are required (Dunn 2001; Lebovka and others 2001, 2002). Many studies investing the effects of moderate electrical field (MEF) treatments have found that treatments at about 100 V/cm can induce tissue damage through membrane breakdown (Wang and Sastry 2002; Lebovka and others 2007). Although PEF treatments have been successfully applied for microbial inactivation in liquid food systems, its application in solid food systems, such as plant tissues, is rather limited due to the lack of knowledge about the effects of PEF on cell structure. The limitations to development of fundamental knowledge on PEF effects in solid food include the structural complexity and heterogeneity of the solid food. Since PEF may cause undesirable changes in foods, it is necessary to fully characterize tissue changes as a function of the electrical field strength and the number of pulses applied. Determination of the critical electric field strength, or threshold, at which changes in plant tissue can be observed, is important to understand changes in tissue properties. Angersbach and others (1999) have suggested that pulsed electrical field treatment of plant tissues can initiate separate membrane breakdowns of the plasma membrane and the tonoplast membrane, giving rise to 2 possible critical electrical field strengths. However, they did not present data to support their hypothesis.

In this study, the effects of PEF on the integrity, texture, and chemistry of onion tissue in 2 different cultivars, Sabroso and Ranchero, were investigated. Changes in the onion tissue's electrical, chemical, and physical properties were determined using electrical characteristics (conductivity disintegration index), ion leakage, alliinase enzyme activity, and physical (texture) changes in the onion tissue subjected to PEF. By measuring different properties of the tissue, the effect of PEF on the properties can be correlated to the structural changes in the tissue to determine if there is more than 1 critical field strength. The critical electric field strengths, at which changes in plant tissue can be observed, are determined at a fixed numbers of pulses. We fixed the pulse width at 100 μs, which is in range of commonly examined pulse widths (10 to 1000 μs) for PEF treatment at moderate electric field strength (Bazhal and others 2001; Lebovka and others 2001, 2002; Arevalo and others 2003), so over results could be compared to the others.

Materials and Methods

Sample preparation

There is no single onion cultivar available year round, thus preliminary studies were carried out to obtain cultivars with similar characteristics. Yellow cultivars were selected since they are the most commonly used in the processing industry. Sabroso and Ranchero, long day Spanish yellow hybrid onions (*Allium cepa*) were obtained from Gills Onions (Oxnard, Calif., U.S.A.) and kept in cold storage at 4 °C for less than 3 mo. Onion bulb size, which is a measure of maturity, was controlled at approximately 3.5 inches in diameter. Both onion cultivars have similar physicochemical properties including a single center, globular shape, and medium to high pungency (that is, pyruvate content). Prior to sampling, onion bulbs were equilibrated to room temperature (approximately 25 °C) for 2 h before removing the papery outer scales and the first fleshy scale. The second fleshy scale was cut into 2 cm diameter disks along its mid section with a core borer. The inner epidermal cell layer was removed and the onion disks were rinsed with deionized water. Excess water was removed by gentle blotting with clean paper towels. To ensure good contact between electrodes and onion tissues, samples with thicknesses of approximately 3 mm were carefully selected to match the depth of the well in the sample holder. The average sample disk thickness was obtained by measuring the sample thicknesses at 5 different locations around the disk periphery with digital caliper (Model CD-6B, Mitutoyo Corp., Tokyo, Japan).

Pulsed electric field (PEF) treatments

The plexiglas sample holder is shown in Figure 1. The cylindrical sample holder consists of a top and bottom chamber, and the bottom chamber has a well (gap) of a specific depth. The top chamber is assembled with a 2 cm diameter flat stainless steel electrode. The well of the bottom chamber, used in these

studies, is 0.3 cm deep and 2 cm in diameter and has a flat stainless steel electrode fixed inside the bottom. An onion sample of the same thickness is placed between the 2 electrodes of the sample chambers with the convex plane facing down. Isotonic solution is added to replace the air inside the chamber, and to allow for better contact between the sample and electrodes. There is a hole in the bottom chamber to provide an overflow exit for excess air and solution. To ensure an airtight condition, o-ring gaskets are present between the top and the bottom electrode assemblies and a constant clamping force is applied to the sample holder using an Arbor press with a fixed deadweight. Note that the sample was not pressurized because of the presence of a small hole to allow air and excess solution to be pushed out to the atmosphere.

Pulsed electric field treatments were carried out using the system shown in Figure 2. The PEF system consists of a high voltage power supply (PowerPAC HV, Bio-Rad, Hercules, Calif., U.S.A.), a function generator (model 33220A, Agilent, Santa Clara, Calif., U.S.A.), which is used for manipulating the pulse shape, width, frequency and number, a PEF generator, a sample holder and an oscilloscope (model TDS1012B, Tektronix, Beaverton, Oreg., U.S.A.) for signal monitoring. The PEF generator provides monopolar positive pulses of rectangular shape with a pulse width $t_i = 100\ \mu s$ and pulse frequency $f = 1$ Hz. Experiments were carried out using electric field strengths (E) between 67 and 333 V/cm (that is, applied potentials of 20 to 100 V/0.3 cm) with number of pulses $n = 1$ to 100 pulses. The total treatment time is $t_{PEF} = nt_i$ (s).

Electrical characteristics and conductivity disintegration index determination

The electrical characteristics of onion tissue before and after PEF were measured with a LCR meter (model 4284A, Hewlett-Packard, U.S.A.) at a frequency of 20 Hz. The applied potential across the sample was set at 1 V. The measurement parameters included resistance R (Ω), impedance Z (Ω), and conductance G (Siemens). Determination of electrical characteristics was replicated at least 3 times per treatment.

The degree of tissue damage is obtained from the electrical conductivity disintegration index Z^* (Lebovka and others 2002)

$$Z^* = \frac{\sigma - \sigma_i}{\sigma_d - \sigma_i} = \frac{(G - G_i)^* L/A}{(G_d - G_i)^* L/A} = \frac{G - G_i}{G_d - G_i} \qquad (1)$$

where, σ is the measured electrical conductivity (Siemens/cm), L is the sample thickness (m), A is cross-sectional area of the sample (m^2), L/A is the cell constant (cm^{-1}), and the subscripts "i" and "d" refer to the conductivities of intact and completely

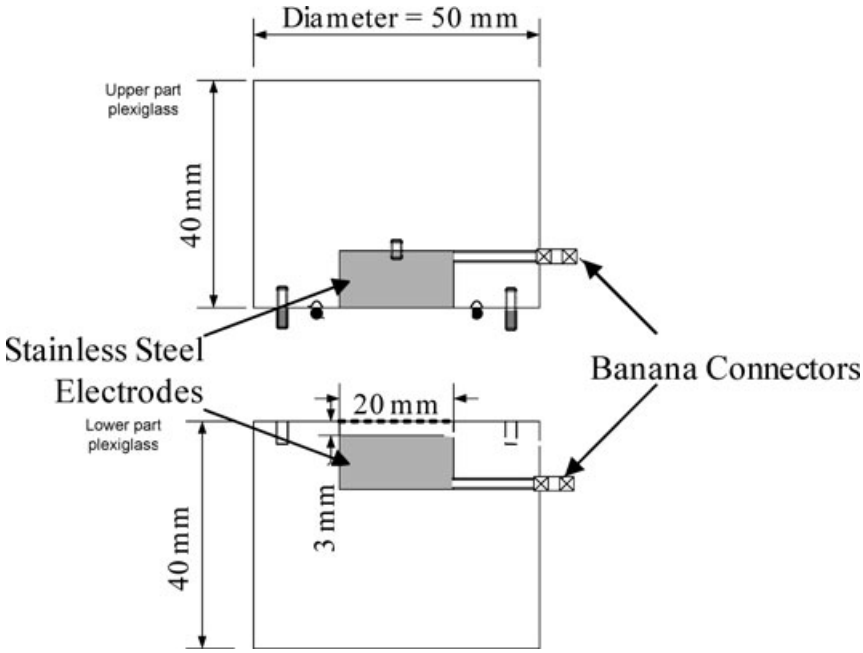

Figure 1–PEF sample holder.

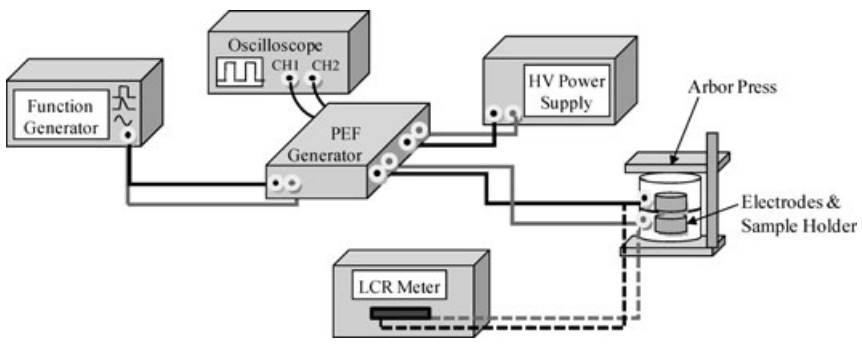

Figure 2–Schematic diagram of the pulsed electric field treatment system.

ruptured tissue, respectively. Complete rupture of the onion tissue was accomplished by 2 cycles of freezing (−18 °C) and thawing at ambient temperature (Palta and others 1977a, 1977b; Gonzalez 2009). From Eq. (1), $Z^* = 0$ for intact tissue (control, no PEF) and $Z^* = 1$ for completely ruptured tissue.

Ion leakage determination

After PEF, control and treated onion disks were covered with damp tissue paper to avoid moisture loss (Saltveit 2002) and kept overnight in the refrigerator. Only the samples evaluated for electrolyte leakage were kept refrigerated overnight. All other measurements were taken immediately after the PEF treatments. For determination of ion leakage (the electrolyte concentration in solution), samples were equilibrated at room temperature for 1 h. Two onion disks were placed into a 50 mL centrifuge tube (28 mm outside diameter and 115 mm long) containing 20 mL of an isotonic solution (0.2 M mannitol) pre-equilibrated at 25 °C. Solid/liquid ratio was approximately 1 : 10.6 by volume (that is, volume of 2 onion pieces = 1.88 cm^3/volume of bathing solution = 20 cm^3). Onion osmolality was determined from preliminary experiments (Saltveit 2002; Gonzalez 2009). The electrical conductivity (σ) of each sample, maintained at 25 °C in a constant temperature water bath, was measured using a conductivity meter (Accumet portable AP65, Fisher Scientific, Singapore) periodically for up to 300 min. The interval between measurements was 5 to 15 min at the beginning of the run and longer, 60 min, towards the end. Between measurements, the rack of tubes was agitated at the level 4 rate setting on a shaking circulating water bath (Model SWB1122A-1, Lindberg, U.S.A.). After the final measurement, the centrifuge tubes were capped and placed in a freezer overnight at −18 °C. The next day, the frozen samples were removed from the freezer and allowed to warm to room temperature, 25 °C. After a second overnight freeze/thaw cycle, maximum tissue damage was created (Gonzalez 2009; Milczarek and others 2009) and a conductivity measurement was taken. The total electrolyte presented in the tissue was used as the "total conductivity." Ion leakage at time t was calculated as a percentage of total conductivity of the sample, as shown in Eq. (2).

$$\%\ \text{Conductivity}(t) = \frac{\text{Conductivity}(t)}{\text{Total Conductivity}} \times 100. \qquad (2)$$

To determine the effect of PEF treatments on percent ion leakage, 2 methods of data interpretation were employed: (1) comparison of % conductivity at 300 min, and (2) comparison of ion leakage kinetic parameters.

To compare ion leakage kinetics, data were fitted according to the following models (Murray and others 1989; Saltveit 2002):

$$y = \frac{a\,t}{(b + t)} \qquad (3)$$

and

$$y = C_0(1 - e^{-kt}), \qquad (4)$$

where y is % conductivity of the sample and t is a specific time of measurement for both equations. In the first model (Michaelis–Menten Equation), the parameter a is the asymptote that represents the maximum percentage of total conductivity reached and parameter b is the time to reach 50% of the maximum conductivity value (1/2 time). In the second model, C_o is the y-intercept of a linear equation fitted to the linear portion of the data at longer incubation times, that is, from $t = 45$ to 240 min, and k is the rate of ion leakage to the medium (Saltveit 2002). The experimental data were fitted with a nonlinear procedure (SAS 9.1, SAS Institute Inc., Cary, N.C., U.S.A.). The best model was selected based on the minimum standard error of the model and the effect of PEF on the model parameters was analyzed using Duncan's multiple range test ($P < 0.05$).

Alliinase activity

The loss of cell integrity and subsequent action of the alliinase enzyme were determined by the amount of enzymatically formed pyruvate in the onion tissue. Pyruvate was assayed using DNPH (2, 4-dinitrophenylhydrazine; Sigma-Aldrich, St. Louis, Mo., U.S.A.) following the procedure of Anthon and Barrett (2003). Following treatment, PEF and control (no PEF) samples were left at room temperature for 1 h to allow the reaction between alliinase and S-alk-(en)yl-L-cysteine sulphoxides to take place and for formation of the product, pyruvate.

Two methods of sample preparation were employed to meet the following objectives: (1) to measure the residual activity of alliinase remaining in the onion tissue after PEF, followed by complete homogenization by grinding the tissue with a mortar and pestle, and (2) to measure the impact of the PEF on cell integrity by measuring the amount of pyruvate formed after PEF (Gonzalez 2009). The amount of endogenous pyruvate initially present was measured in the control sample. The additional amount of pyruvate formed was used as an indicator of cell membrane damage as a result of PEF.

Residual alliinase activity. To determine residual alliinase activity, samples were prepared by homogenizing approximately 1 g of onion tissue with distilled water (1 : 1). The homogenate was allowed to sit for 15 min so that alliinase could react with the cysteine sulphoxides, after which 2 mL of 1 M TCA (Sigma-Aldrich) were added. The homogenate was centrifuged for 5 min at 16.1 relative centrifuge force (Centrifuge 5415D, Eppendorf, Germany) and the reaction and pyruvate analysis were carried out as described by Gonzalez (2009).

Alliinase activity during PEF processing. To measure alliinase activity due to PEF processing and not as a result of tissue homogenization, control, and PEF onion samples were homogenized with 1 M TCA, to inactivate alliinase, and distilled water in the ratio of 1 : 2 : 1 (sample : TCA : distilled water). The homogenate was centrifuged and then analyzed as described by Gonzalez (2009). However, the sample weight was doubled due to lower pyruvate content and the volumes of water and aliquots of 1.5 N NaOH (Fisher Scientific, Pittsburgh, Pa., U.S.A.) were adjusted accordingly.

Texture measurement

Puncture tests were performed using a 2 mm diameter flat-tipped cylindrical probe with a Texture Analyzer (model TA.XT2, Texture Technologies Corp., Scarsdale, N.Y., U.S.A./Stable Micro Systems, Godalming, Surrey, U.K.) and a 25 kg load cell. The tests were performed to 90% deformation of the original onion scale thickness and the test speed was set to 1 mm/s. Texture parameters measured were maximum force (N), gradient (from initial point to 20% maximum force) (N/mm), and number of peaks (threshold force = 0.15 N). At least 15 disks of 0.3 cm thickness and 2 cm diameter were used for each data point.

Statistical analysis

Data were analyzed using both SAS 9.1 (SAS Institute Inc.) and SPSS 11.5 (SPSS Inc., Chicago, Ill., U.S.A.) package programs. A 2-factor factorial model was developed to test for the interaction effect between the 2 PEF parameters of interest, E and n. Since the interaction was significant ($P < 0.05$), the effect of each parameter was investigated separately. The effect of electric field strength was determined for a fixed number of pulses. The critical electric field strength threshold (E_c) is defined as the maximum electric field strength that the tissue can withstand before any changes in tissue properties could be observed. One-way ANOVA and Duncan's multiple range tests were used for determination of the differences between the PEF conditions ($P < 0.05$). The probability that the property of onion tissue treated at $E < E_c$ is different from the control is less than 5%.

Results and Discussion

Effect of PEF on electrical characteristics and conductivity disintegration index (Z^*)

For the Sabroso onion cultivar, the average conductivities of intact (G_i) and ruptured (G_d) onion tissues are 225 and 1620 μSiemens, respectively. The degree of tissue rupture caused by PEF was calculated using Eq. (1). The conductivity disintegration index Z^* value is plotted against the electric field strength as illustrated in Figure 3. Following single pulse PEF, the electrical characteristics (Supporting Information A1) and Z^* value of onion samples remain unchanged at all field strengths ($E = 0$ to 333 V/cm). However, following 10 and 100 pulses of PEF, higher Z^* values are observed with increasing field strength, as shown in Figure 3. Pulsed electrical fields applied at $E \geq 133$ V/cm for both 10 and 100 pulses significantly affect the Z^* value of onion tissue ($P < 0.05$); the Z^* value increases with increasing field strength at E levels between 67 and 333 V/cm. Increasing the pulse number leads to a higher Z^* or degree of tissue damage, at the same applied electric field strength. Fincan and Dejmek (2002) reported similar results, for example, no effect on membrane permeabilization, with a single PEF pulse treatment of onion epidermis. These researchers found that epidermal cells were still intact if one PEF pulse was applied for the same electrical field range as ours. The threshold electric field strength for membrane rupture that they determined is at $E_c = 350$ V/cm.

An increase in Z^* value reflects an increase in conductance G. Increased G is an indication of increased permeability of the cell membrane and loss of plant tissue integrity, which results from cell membrane permeabilization or breakdown caused by PEF (Knorr and Angersbach 1998; Lebovka and others 2000; Kulshrestha and Sastry 2006). De Andrade and others (1999) used measurement of the electrical conductivity of bean seeds to evaluate the degree of mechanical damage. Seeds of *Phaseolus vulgaris* were mechanically damaged at velocities of 10, 13, or 16.5 m/s. The electrical conductivity measurements agreed well with the degree of mechanical damage.

The critical electric field strength threshold (E_c) above which application of PEF causes tissue rupture is 67 V/cm for both 10 and 100 pulse PEF treatments. Note that the E_c value for 100 pulse PEF treatments could be lower than 67 V/cm but this is the lowest electric field strength possible with the PEF system used in this experiment. The values of Z^* increase, but the changes appear to have plateaus both in the mid-range and the upper range of E (Figure 3). The electric field strength thresholds that contribute to a second significant change in electrical characteristics are 200 and 133 V/cm for 10 and 100 pulse treatments, respectively. These results suggest that PEF treatments above these E_c levels result in tonoplast membrane breakdown and this will be discussed in the section "The Determination of Critical Electric Field Strength Threshold: Plasma and Tonoplast Membrane Breakdown."

Increasing the pulse number causes more damage to onion tissue as illustrated in Figure 4. PEF treatment at for 10 pulses causes a significant different in Z^* value, which indicates significant tissue damage ($P < 0.05$). For up to 100 pulses, PEF treatment at $E =$ 333 V/cm results in a Z^* value of 0.58 ± 0.27, while a Z^* value of about 0.95 is observed for PEF treatment at $n = 200$. Above 200 pulses, PEF treatment at $E = 333$ V/cm causes no significant change in Z^* value since the degree of tissue damage reaches its maximum limit ($P < 0.05$). This result follows the same conclusions by other studies (Lebovka and others 2001, 2002; Arevalo and others 2003) suggesting that there is a saturation threshold

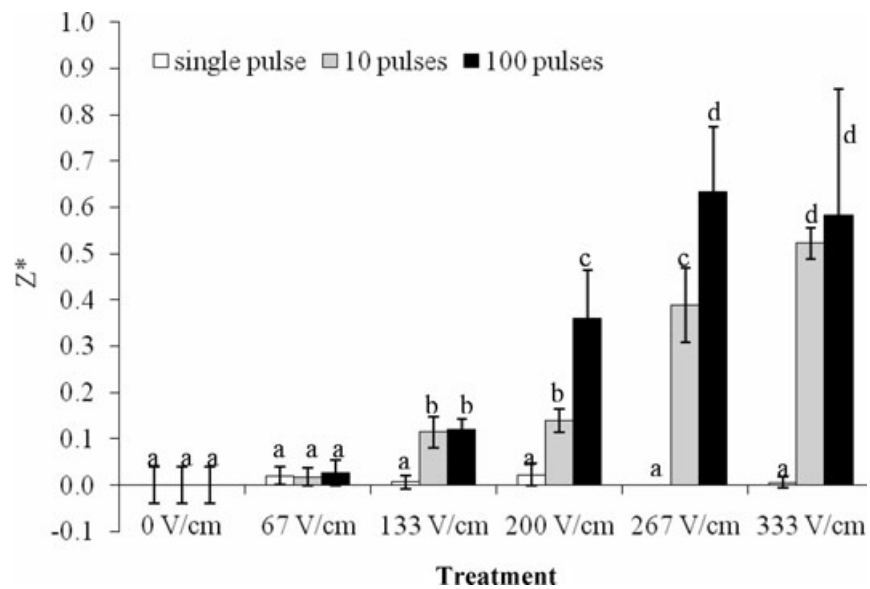

Figure 3–Effect of electric field strength *E* and pulse number *n* on electrical conductivity disintegration index *Z of onion tissues. Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$) and the comparisons are presented separately for each pulse number.**

beyond, which PEF has no effect on membrane breakdown. Depending on the applied electric field strength, the threshold of pulse number can be different. A slight increase in electric field strength results in a dramatic decrease in number of pulses required to get the same degree of tissue damage; for example, increasing E from 200 to 267 V/cm can remarkably reduce the number of pulses required to obtained $Z^* = 0.4$ from 100 to 10 pulses (Figure 3d). This finding suggests that higher field strengths result in lower thresholds of pulse number. Arevalo and others (2003) reported that up to 50% of the total change in electrical conductivity occurred during the first 5 pulses of PEF treatment at $E = 0.75$ and 1.5 V/cm for apple tissue at pulse durations of 100 to 300 μs.

Effect of PEF on ion leakage profile

The ion leakage profiles of onion tissues subjected to different combinations of electric field strengths ($E = 0$ to 333 V/cm) and pulse number (n = 10, 20, and 100) were investigated and an example of the $n = 100$ pulses results is shown in Figure 5. The relationship between electrical conductivity and time has been shown to follow an asymptotic curve (Saltveit 2002). Initially, the rate of ion leakage increases rapidly and then gradually levels off. Untreated samples also show an increase of conductivity (approximately 10%) over time, most likely due to leakage of electrolytes from cells cut during sampling and/or from the apoplastic region of the tissue. For all treatments (data not shown) the change in % conductivity reaches a plateau after 240 min of ion leakage measurements, that is, steady-state conductivity was obtained. Therefore, while the entire ion leakage profiles were determined for each treatment, only the final conductivity values at 300 min are further discussed.

Figure 6 indicates that a single pulse treatment has no significant effect on % conductivity ($P < 0.05$), which is consistent with the electrical characteristics results discussed previously. As mentioned previously, single pulse treatment causes no significant effect in electrical characteristics of onion tissue. Thus, ion leakage experiments were done solely on onion treated at maximum E of

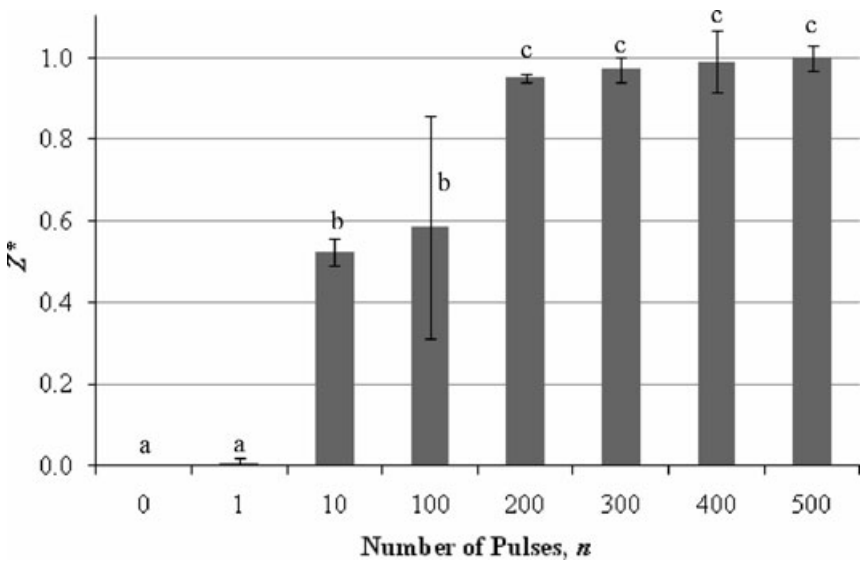

Figure 4–Effect of number of pulses ($n = 0$ to 500) on electrical conductivity disintegration index Z^* of onion tissues treated by PEF at $E =$ 333 V/cm. Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$).

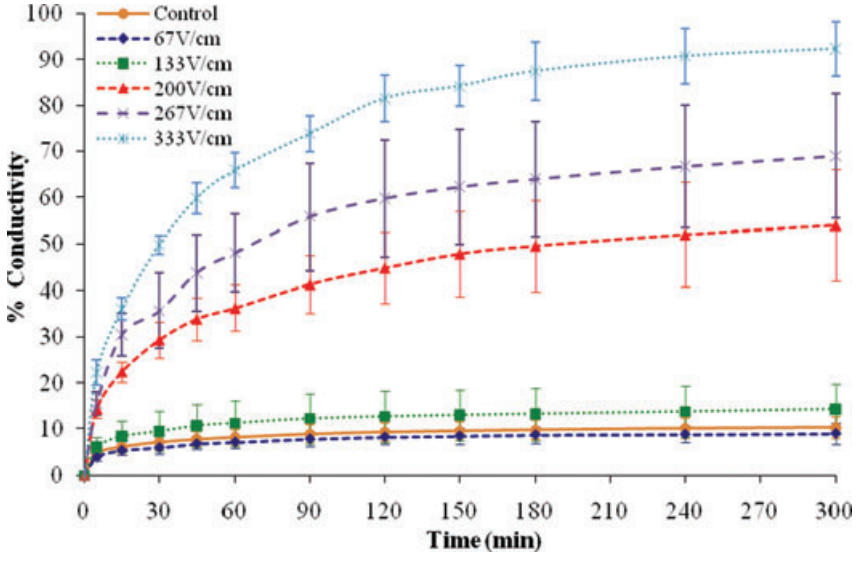

Figure 5–Ion leakage profiles of onion samples subjected to PEF treatments of 100 pulses at various electric field strengths (that is, $E = 0$ to 333 V/cm).

333 V/cm to confirm the result and the results show no significant effect of a single pulse for all electric field strengths up to 333 V/cm. For 10 and 20 pulses, application of PEF results in a similar effect on the conductivity, and the threshold of change is observed at $E_c = 200$ V/cm. PEF at $E \geq 267$ V/cm resulted in a significant change as compared to the control ($P < 0.05$). For 100 pulse PEF treatments, the threshold for cellular integrity change is at $E_c =$ 133 V/cm. PEF at $E \geq 200$ V/cm resulted in a significant change in the conductivity compared to the control ($P < 0.05$).

It is rather complicated to explain the electrolyte leakage from plant tissues with a diffusion approach. Ion transport in plant tissues may also involve convection. Further, contributions to ion transport occur via 2 different paths, that is, the apoplast and the symplast, which yield different rates of ion transport (Kocheva and others 2005). The empirical equations were generally used to fit the electrolyte leakage data and determine the overall ion transfer rate (Murray and others 1989; Saltveit 2002; Gonzalez 2009). Although, specific diffusion coefficients could not be determined, it is unquestionable that the percent conductivity supplied valuable information for the membrane function and the overall picture of the physiological status of the plants (Gavuzzi and others 1997; Rizza and others 2001).

The kinetics of ion leakage were analyzed by fitting the ion leakage data to the proposed empirical models. Comparing the sum square error of each parameter obtained from the 2 proposed models, the first model: $y = \frac{at}{(b+t)}$ fits the ion leakage profile the best. Table 1 summarizes the conductivity (a) and the half time to reach maximum conductivity (b) obtained from onion tissues for different PEF conditions. For 10 and 20 pulses, the final conductivity is the same as the control ($P < 0.05$), up to the threshold electric field strength of 200 V/cm. PEF at E above the threshold level yield at least 4-fold higher conductivity values compared to the control.

Application of 100 pulses PEF at $E \geq 200$ V/cm results in a significant increase in the conductivity ($P < 0.05$) compared to the control. Following application of 100 pulses at $E = 333$ V/cm, the conductivity reaches levels of 100%, or complete tissue rupture, within the 300 min incubation period. Statistical analysis shows that significant changes in ion leakage, as determined by either the model a or b parameters, are the same.

Calculation of ion leakage kinetic parameters allows for the determination of the permeability of onion cells after PEF. Electrolyte leakage has long been used as a means to evaluate the intactness and permeability of cell membranes (Murray and others 1989; Vasquez-Tello and others 1990). In general, the direction of ion transport will follow the path from a high concentration inside the cell to a lower concentration outside in the mannitol solution. The efflux of electrolytes is driven by passive diffusion, convection, and electrical migration, while electrolyte influx is due to active transport. Loss of tissue integrity, as indicated by ion leakage, may result from either an increased efflux due to damage to the plasma membrane, or a decreased influx due to damage to the active transport system (Palta and others 1977a).

Fitting the ion leakage profile to the kinetic model, the % conductivity and half time values can be obtained. These parameters are used to identify the threshold levels of electric field strength at which the integrity of the onion tissue is retained. For the 10 and 20 pulse PEF treatments, the threshold is 200 V/cm, while a threshold of 133 V/cm is obtained for the 100 pulse treatment. Increasing pulse numbers from 10 to 100 yields a lower electric field strength threshold. PEF treatments at E above the threshold level resulted in loss of membrane integrity, in which case the plasma membranes no longer acted as barriers to ion transport, as indicated by increasing conductivity values.

Same level of magnitude of electric field strength threshold was also reported by Angerbach and others (2000). They studied the effects of PEF on cell membranes in potato, apple and fish tissues, as well as plant cell suspension and observed a slight membrane breakdown phenomenon after a single pulse at an E_c of 150 to 200 V/cm was applied. Significant membrane breakdown was observed when the treatment at $E = 400$ to 800 V/cm was applied directly to the cell systems.

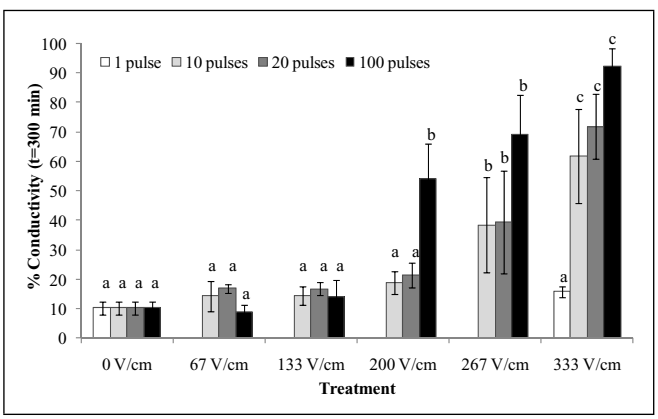

Figure 6–Effect of electric field strength (E) and pulse number (n) on % conductivity of PEF-treated onion in 0.2 M mannitol solution at $t = 300$ min. Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$) and the comparisons are separately for each pulse number.

Effect of PEF on alliinase activity: pyruvate formation

As shown in Figure 7, the amount of pyruvate formed during PEF increases with increasing values of electrical field strength and pulse number. The pyruvate content of control samples was

Table 1–Comparison of the % conductivity (a) and the half time (b) to reach maximum conductivity value obtained from onion tissues treated by different PEF treatment conditions.

Electric field strength, E (V/cm)	10 pulses		20 pulses		100 pulses	
	Conductivity (%)	1/2 Time (min)	Conductivity (%)	1/2 Time (min)	Conductivity (%)	1/2 Time (min)
0 (Control)	10^{a}	10^{a}	10^{a}	10^{a}	10^{a}	10^{a}
67	14^{a}	10^{a}	17^{a}	9^{a}	9^{a}	10^{a}
133	14^{a}	9^{a}	17^{a}	13^{ab}	14^{a}	10^{a}
200	19^{a}	9^{a}	21^{a}	11^{ab}	57^{b}	27^{b}
267	39^{b}	20^{ab}	40^{b}	18^{b}	73^{c}	27^{b}
333	68^{c}	31^{b}	77^{c}	29^{c}	100^{d}	28^{b}

Values with a common letter do not differ significantly ($P < 0.05$).

0.39 ± 0.16 μmoles/g-tissue, most likely due to enzymatic reactions in cells cut by the sample preparation. The maximum pyruvate content measured in PEF-treated onion samples is 5.91 ± 0.23 μmoles/g tissue (data not shown). None of the PEF treatments results in more than 25% of the potential pyruvate content, nor did any treatments cause alliinase inactivation.

For 10, 20, and 100 pulses PEF at the highest electric field applied, 333 V/cm, the pyruvate content of samples increases from 0.39 ± 0.16 to 0.76 ± 0.08, 0.88 ± 0.28, and 1.32 ± 0.46 μmoles/g-tissue, respectively. The critical electric field strengths determined are 200 V/cm and 267 V/cm for 100 and 10 pulse treatments, respectively. At 333 V/cm, the effect of pulse number (10, 20, and 100) on pyruvate content is not significant ($P \leq 0.05$) but there is variability in these results. The amount of pyruvate formed after a 10 pulse treatment at 333V/cm is significantly higher than at 200 V/cm, but not different from 267 V/cm ($P < 0.05$). A 333V/cm application for 100 pulses produces significantly more pyruvate than the 133 V/cm treatment ($P < 0.05$).

Pyruvate is formed in onion tissue as the result of the hydrolysis of S-alk(en)yl cysteine sulphoxides, which are flavor precursors present in the cell cytoplasm, by the enzyme alliinase, which is initially located in the vacuole of intact cells (Lancaster and Collin 1981; Anthon and Barrett 2003). When the tonoplast membrane surrounding the vacuole is ruptured as a result of PEF treatment, the enzyme comes into contact with its substrate and the reaction producing pyruvate. The enzyme and its amino acid substrate are separated *in vivo* and only react on rupture or wounding of onion cells (Lancaster and Collin 1981). Pyruvate is a good measure of the loss in tonoplast membrane integrity and the subsequent action of alliinase, hence this method was chosen to evaluate the effect of PEF on tonoplast membrane rupture rather than plasma membrane rupture.

The highest amount of pyruvate forms after 100 pulses at 333 V/cm, but this is still lower than the maximum possible amount of pyruvate obtained after homogenization of samples with water for a 10-min reaction time. It is hypothesized that during PEF, the tonoplast membrane ruptures but the cell walls remain rigid and protects the cell structure. This would lower the diffusion of different cell components to each other in the allocated reaction time. For mechanical tissue homogenization, all of the cell components are decompartmentalized, allowing for interaction of the enzyme and substrate.

Gonzalez (2009) studied the effect of high pressure and thermal treatments with onion tissues and determined the pyruvate formation occurs at the 300 MPa high-pressure and 60 °C thermal treatments. Due to the enzyme inactivation during these processes, it was mentioned that the method has limitations for explaining accurately the loss of membrane integrity in their study.

Effect of PEF on texture parameters

Figure 8 illustrates the effects of PEF on the texture of onion tissues in terms of changes in maximum force, gradient, and number of peaks. There is no significant difference ($P < 0.05$) in maximum force (Figure 8A) for 10 and 20 pulses. However, for 100 pulses at higher electric field strengths, the maximum force required to rupture the tissue increases with increasing electric field strength. Maximum force, which represents the hardness (Bourne 2002) of the tissue, is 6.88 ± 0.92 N for the control and 9.52 ± 1.69 N for samples treated with 100 pulses at 333 V/cm. Maximum force of the samples treated for 100 pulses at 333 V/cm is significantly higher than whole lower levels of E (V/cm) treated samples ($P < 0.05$).

An increase in the maximum force may be caused by pectin cross-linking or may be due to the compaction of cell layers within the tissue (Gonzalez 2009). Further, pectin methyl esterase activation may result as a consequence of cell membrane rupture and leakage between compartments, changing the environment of the enzyme (Anthon and Barrett 2006), and potentially causing calcium binding and improved hardness.

The gradient and number of peaks in the force deformation curves indicates changes as due to PEF. Gradient values decrease with increasing electric field strength and number of pulses, and PEF-treated samples have fewer peaks as compared to the control samples. The 10 and 20 pulse treatments have changes in both gradient and number of peaks for $E > 200$ V/cm, while the critical electric field strength for 100 pulses is 133 V/cm (Figure 8B and 8C). Gradient and number of peaks decreased significantly after 10 and 20 pulses at $E \geq 267$ V/cm and 100 pulses at $E \geq 200$ V/cm ($P < 0.05$).

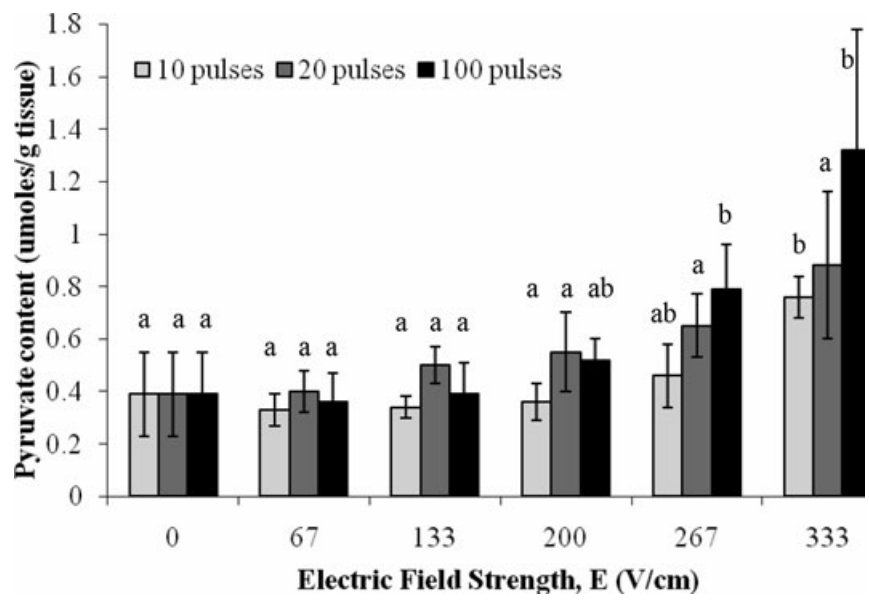

Figure 7–Effect of electric field strength (*E*) and pulse number (*n*) on alliinase activity and pyruvate formation. Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$) and the comparisons are separately for each pulse number.

Peaks present in the texture profile following the maximum force may arise from the puncture probe passing through layers of intact cells (Gonzalez 2009). Intact plasma and tonoplast membranes form a boundary for the development of cellular turgor pressure, and this together with the plant cell wall imparts rigidity and firmness to plant tissues. The gradient is correlated to the stiffness and a larger number of cells with intact membranes have greater stiffness (Bourne 2002; Gonzalez 2009). The gradient has also been correlated to the membrane integrity of onion cells (Gonzalez 2009). It was also reported that there is strong correlation between percentage of stained area (viable cells), the number of peaks present, and the initial slope (gradient) for both high-pressure and thermally processed onions, indicating that these parameters are related to the viability of the cells and the state of the membranes (Gonzalez 2009).

Application of PEF of sufficient strength and pulse number results in a high degree of disintegration of plant cell membranes and for removal of the turgor component of texture (Lebovka and others 2003). Lebovka and others (2004) studied the effect of pulsed electric field treatments on textural properties of carrots, potatoes, and apples; also they mentioned PEF causes a nonthermal rupture of cellular membranes and decrease of the turgor components of cells. It has been suggested that fruit firmness, in common with the biomechanical properties of most plant tissues, is influenced by cellular turgor pressure (Shackel and others 1991). Products lose their firm/crisp texture on heating because of loss of turgor (Szczesniak 1998).

When membrane integrity is lost in onion tissue, the gradient, and number of peaks after the maximum force change significantly after the threshold electric field and, consequently, these measurements can be used for evaluation of membrane integrity in PEF-treated samples and for differentiation of the effect of different PEF treatment conditions.

Determination of critical electric field strength threshold: plasma and tonoplast membrane breakdown

The critical electric field strength thresholds determined for each number of pulses applied are summarized in Table 2. A single pulse treatment, in the range of E investigated, has no significant effect on onion tissue integrity, as determined by all methods. Increasing the number of pulses decreases the electric field strength required to reach the critical electrical threshold value. The E_c values determined using electrical characteristic criteria are lower than the others and may reflect initial changes in plasma membrane integrity that could not be detected by other measurement methods. However, changes in electrical characteristics are not specific to plasma or tonoplast membranes. As reported in many studies, a change in electrical conductivity is a direct indication of cell membrane integrity loss (Knorr and Angersbach 1998; Lebovka and others 2002; Arevalo and others 2003). Unless the vacuole membrane is ruptured, the breakdown of plasma membrane alone will not significantly change ion leakage and texture properties of onion tissue. Note that the tonoplast membrane surrounds the vacuole, which occupies up to 80% to 90% of the total volume of the mature cell (Taiz and Zeiger 2006).

As mentioned previously, the electric field strength thresholds that contribute to a second significant change in electrical

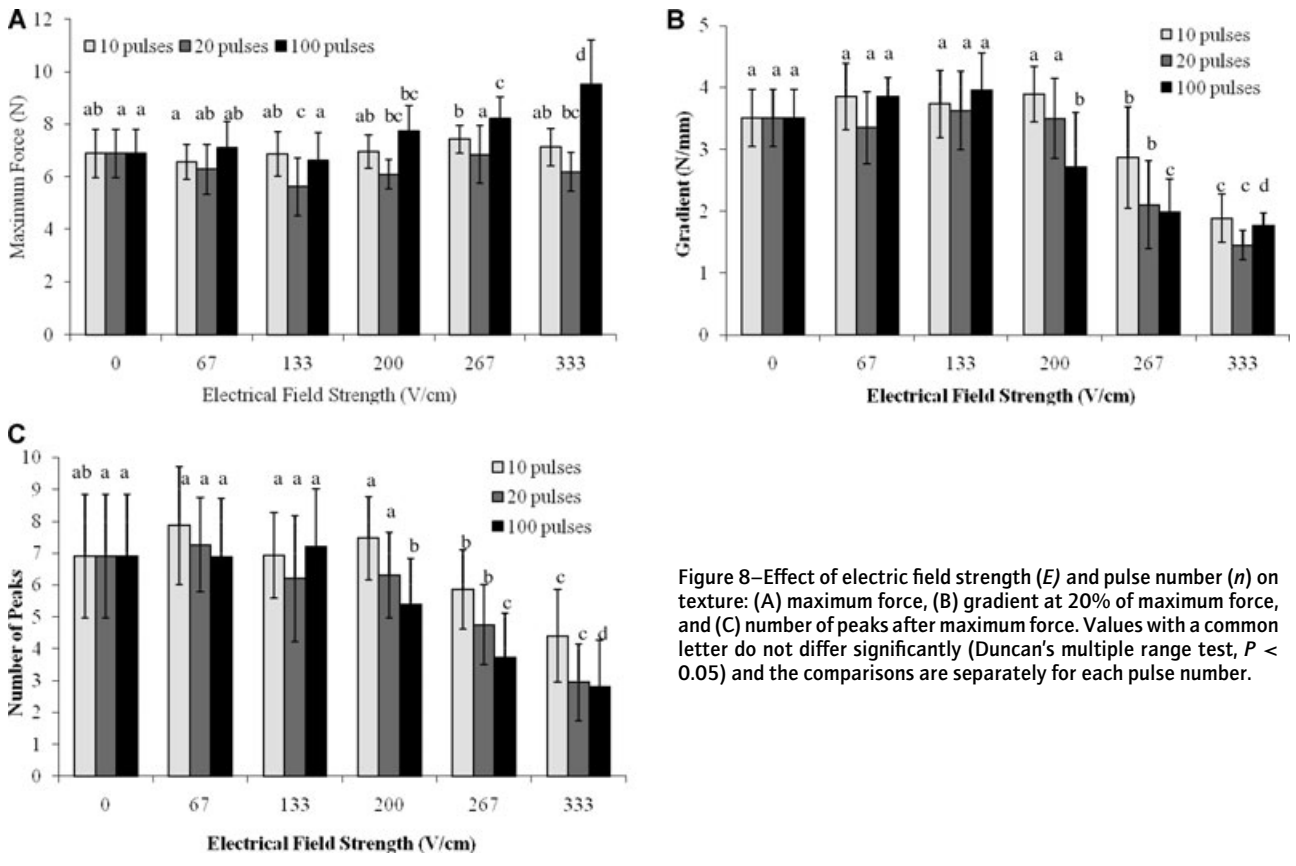

Figure 8–Effect of electric field strength (*E*) and pulse number (*n*) on texture: (A) maximum force, (B) gradient at 20% of maximum force, and (C) number of peaks after maximum force. Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$) and the comparisons are separately for each pulse number.

Table 2–Summary of critical electric field strength thresholds E_c (in V/cm) based on different criteria. Values indicate the electric field strength at which a significant change in the specific analytical criteria measured (electrical characteristic, ion leakage, alliinase, and texture) occurred (Duncan's multiple range tests, $P < 0.05$). Plasma membrane rupture is indicated by the first value; values in parentheses are secondary thresholds, which suggest tonoplast membrane rupture.

		Number of pulses			
Criteria	**Membrane indicated**	**1**	**10**	**20**	**100**
Electrical characteristics					
Resistance, *R*	Not specific–-plasma and tonoplast membranes	—	67 (200)	n/a	67 (133)
Impedance, *Z*		—	67 (200)	n/a	67 (133)
Conductance, *G*		—	67 (200)	n/a	67 (133)
Disintegration Index, Z^*		—	67 (200)	n/a	67 (133)
Ion leakage	Not specific–-plasma and tonoplast membranes	n/a	200	200	133
Alliinase activity	Tonoplast membrane	n/a	267	—	200
Texture properties					
Max force	Not specific–-plasma and tonoplast membranes	—	—	—	—
Gradient		n/a	200	200	133
Number of peaks		n/a	200	200	133

"—" indicates no significant difference among all *E* levels; (n/a) indicates no experiment carried out for this particular method.

characteristics are 200 and 133 V/cm for 10 and 100 pulse treatments, respectively (Figure 3; Table 2). These secondary thresholds are similar to the E_c values determined by ion leakage and texture measurement. These results are indicative that PEF treatments above these E_c levels result in tonoplast membrane breakdown. Alliinase activity measurement proves to be a good method of detection for tonoplast membrane rupture. However, compared to ion leakage and texture measurements, slightly higher E_c values were determined by this method. This may due to the fact that, above E_c, the tonoplast membrane ruptures but the cell walls remain rigid and impede diffusion of enzyme and substrate to form pyruvate in the allocated reaction time. Our results support the hypothesis proposed by Angersbach and others (1999) that PEF treatment of plant tissues can initiate separate breakdowns of the plasma membrane and the tonoplast membrane, giving rise to 2 critical electrical field strengths.

Conclusions

Results indicate there are significant influences of electric field strength *E* and pulse number *n*; using constant pulse duration of 100 μs and pulse frequency of 1 Hz, on PEF-treated onion tissue integrity, texture, and chemistry. The critical electric field strength is determined for the number of pulses applied. Electrical, chemical, and physical determinations of tissue integrity can be used to differentiate between plasma membrane breakdown and tonoplast membrane breakdown. We report that the different measurement methods utilized in this study give different critical electric field thresholds. Measurement of electrical characteristics could be used to detect the earliest stage of plasma membrane breakdown, while pyruvate formation by the enzyme alliinase is the method that directly indicates tonoplast membrane breakdown. Our results suggest that there appears to be 2 critical electrical field strengths: one for the plasma membrane and a higher one for the tonoplast membrane in agreement with the hypothesis proposed by Angersbach and others (1999). All methods of tissue integrity determination indicate that a single pulse treatment has no significant effect. Increasing the number of pulses decreases the electric field strength required to reach the critical electrical threshold value. The critical field strength threshold for plasma membrane breakdown is 67 V/cm for 10 pulses treatment, and the critical thresholds for tonoplast membrane breakdown are either 200 V/cm for 10 pulses or 133 V/cm for 100 pulses. In addition, a slight increase in electric field strength results in a dramatic decrease in number of pulses required to get the same degree of tissue damage. Thus, one could increase PEF efficiency by using higher field strengths with a lower number of pulses.

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Supporting Information

The following supporting information is available for this article.

A1. Effect of PEF on electrical characteristics (*R*, *Z*, and *G*).

Single pulse PEF has no significant effect on resistance R, impedance Z and conductance G of onion samples ($P < 0.05$). Following single pulse PEF the R, Z and G values remains unchanged at all field strengths ($E = 0$–333 V/cm). However, following 10 pulse and 100 pulse PEF, lower values of R and Z and conversely higher G value are observed with increasing field strength, as shown in Figure A1a to A1c. Pulsed electrical field applied at $E \geq 133$ V/cm for 10 pulses and $E \geq 67$ V/cm for 100 pulses had significant effects on R and Z values of onion tissue ($P < 0.05$), and PEF applications at $E \geq 133$ V/cm for both 10 pulses and 100 pulses significantly affected G value of onion tissue ($P < 0.05$).

Figure A1. Effect of electric field strength E and pulse number n on: (a) resistance R, (b) impedance Z, and (c) conductance G of onion tissues. Values with a common letter do not differ significantly (Duncan's multiple range test, $P < 0.05$) and the comparisons are separately for each pulse number.

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