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Effect of the Ionic Strength of Pulsed Electric Field Treatment Medium on the Physicochemical and Structural Characteristics of Lactoferrin

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Pulsed electric field (PEF) treatment (35 kV cm^{-1} for 19.2 μs using bipolar 2 μs pulses) was conducted on bovine lactoferrin (LF; 0.4 mg mL^{-1}) prepared in simulated milk ultrafiltrate (SMUF), at concentrations between 0.2× and 2× normal strength, with electrical conductivities ranging from 0.17 to 1.04 S m^{-1}. The physicochemical and structural characteristics (LF content by a spectrophotometric and an ELISA method, surface hydrophobicity, electrophoretic mobility, far-UV circular dichroism spectra, and tryptophan fluorescence) of LF dissolved in SMUF of all strengths tested were not changed after PEF treatment. The PEF treatment of LF in 0.2 strength SMUF did not cause the release of LF-bound ferric ion into the aqueous phase, with a concentration of LF-bound iron being the same as that of the untreated LF control (174 μg L^{-1}). However, in treatment media with higher ionic strengths, ferric ion was released from the LF molecule into the aqueous phase. The concentration of LF-bound iron decreased from 174 μg L^{-1} for the LF treated in 0.2 strength SMUF to 80 μg L^{-1} for that treated in double-strength SMUF. The results suggest that the PEF-induced iron depletion of LF does not appear to cause an appreciable conformational change in LF molecules. PEF treatment could be developed as a novel physical way to produce iron-depleted LF, as an alternative to the existing chemical method.

KEYWORDS: Pulsed electric field; lactoferrin; iron release; physicochemical and structural characteristics

INTRODUCTION

Lactoferrin (LF) is an iron-binding protein with a molecular weight of 78 kDa. Each LF molecule has two iron-binding sites, each of which can bind one ferric ion via the ligands between the ferric ion and four amino acid residues (**Figure 1**). The iron saturation level of naturally existing LFs (N-LF) is about 20−30%, but LF can also exist as apo (Apo-LF) or holo (Holo-LF) forms in which the LF molecules are either largely depleted of, or saturated with, ferric ions, respectively (*1*, *2*). Many studies have demonstrated that LF is a bioactive protein with multifunctional activities in addition to iron binding, including antimicrobial, anti-infective, immunomodulatory, and anti-inflammatory effects, and that the iron saturation level affects some of the biological functions of LF (*3*−*5*).

Pulsed electric field (PEF) is a food-processing technology that delivers high-intensity electric pulses (in the range of 1−60 kV cm^{-1}) with short duration (in the range of microseconds) to cause permeabilization of biological cells (*6*). Depending on the treatment conditions applied, PEF can be used to improve the mass transfer of plant or animal tissues during extraction and dehydration processing (0.7−3.0 kV cm^{-1}, 1.0−20 kJ kg^{-1}) or to inactivate

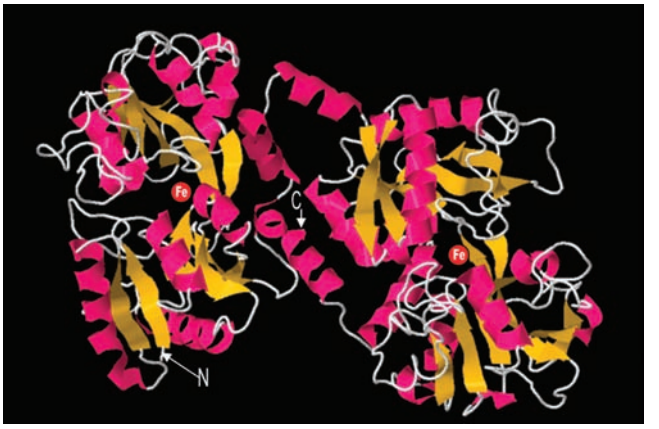

Figure 1. Secondary structure and iron-binding sites of lactoferrin [derived using Jmol via PBD Web site (PBD ID: 1BLF, primary data from Moore et al. (*37*)].

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microorganisms in liquid foods (15−60 kV cm^{-1}, 40−1000 kJ kg^{-1}) to ensure food safety with lower temperatures and shorter heating times than those occurring during conventional thermal pasteurization (*7*). When conducted under conditions sufficient for microbial inactivation, PEF treatment is reported to have little effect on heat-sensitive food components, such as vitamins and proteins (*8*−*10*). However, when proteins are subjected to a more severe PEF treatment, the alteration of protein structures can occur. For instance, Perez and Pilosof (*11*) observed the formation of aggregates and changes in denaturation temperature and enthalpy of β-lactoglobulin and egg white proteins after PEF treatment at 12.5 kV cm^{-1} using up to 10 pulses (specific energy input up to 2465 kJ L^{-1} per pulse). More recently, Zhao et al. (*12*) reported that PEF treatment at 40 kV cm^{-1} for 400 μs did not change the solubility or turbidity of egg white proteins but formed small soluble protein aggregates. It is worth noting that some studies in the literature did not consider the localized temperature increase of the sample during the PEF treatment, and therefore, the observed changes after PEF treatment may be a result of the concurrent thermal effect due to ohmic heating. Nevertheless, it has been proposed that PEF treatment under well-controlled conditions could be a novel way to modify the physicochemical properties or functionality of proteins (*13*).

A PEF treatment process consists of a number of parameters, including electric field intensity, treatment time (determined by pulse width, number of pulses, and flow rate), ionic strength of the treatment medium (affecting the electrical conductivity), pH, and treatment temperature. Few studies are available on the effect of these PEF treatment parameters on LF. De Luis et al. (*14*) reported that the concentration of LF (as detectable by an antibovine LF antibody) in either diluted raw skim milk or diluted whey (0.2 S m^{-1} for both media) was not changed after a PEF treatment at approximately 38 kV cm^{-1} using 50−200 exponential pulses (treatment temperature < 35 °C). The ionic strength of PEF treatment media has been reported to affect the inactivation of *Salmonella* Dublin (*15*) but not to influence that of *Listeria innocua* (*16*) or *L. monocytogenes* (*17*). However, it is still unknown whether the ionic strength of the treatment medium is a factor that contributes to the potential changes in proteins during a PEF treatment. To understand the effect of ionic strength of the PEF treatment medium on LF, the current study investigated the physicochemical and structural characteristics of PEF-treated LF in a model solution with different ionic strengths but with other treatment parameters (i.e., electric field intensity, treatment time, and temperature) kept constant. PEF treatment conditions of 35 kV cm^{-1} for 19.2 μs were used, on the basis of previous work within our research group using the same PEF unit (*20*, *21*) in which these conditions were found to cause the inactivation of the natural microbial flora in raw skim milk.

MATERIALS AND METHODS

Lactoferrin Preparations. Purified bovine LF samples, kindly donated by Dr. J.-P. Perraudin of Biopole S.A. (Les Isnes, Gembloux, Belgium), had a protein content of 98.3% and an iron saturation level of 24.5% (lot DUABIO). Simulated milk ultrafiltrate (SMUF) was prepared using the formulation reported by Jenness and Koops (*18*). The composition of the SMUF consisted of (grams per liter) the following: lactose, 50.00; KH_2PO_4, 1.58; tripotassium citrate, 0.98; trisodium citrate $2H_2O$, 1.79; K_2SO_4, 0.18; $CaCl_2 \cdot H_2O$, 1.32; $MgSO_4$, 0.32; K_2CO_3, 0.30; KCl, 1.08. The concentration of SMUF was adjusted by dilution with deionized water to one-fifth, one-half, or four-fifths of its original concentration or proportionally (1.5× or 2×) increasing the amount of the components in SMUF. The resulting solutions were 0.2, 0.5, 0.8, 1, 1.5, and 2 strength of SMUF with electrical conductivities of 0.17, 0.33, 0.48, 0.56, 0.83, and 1.04 S m^{-1} at 20 °C, respectively. The LF solutions (0.4 mg mL^{-1}, pH 6.7) for PEF treatment were prepared by dissolving LF in the SMUF of different strengths, with gentle mixing using a magnetic stirrer at 4 °C overnight for a complete rehydration of the protein.

PEF Treatment. The laboratory-scale PEF system used in this study was supplied by The Ohio State University (OSU-4, Columbus, OH). Detailed descriptions of the pulse generator have been reported previously (*10*, *19*). The flow cell, connected to the pulse generator, consisted of four cofield treatment chambers, each with a diameter of 0.23 cm and a gap between the electrodes of 0.29 cm. The four treatment chambers were connected in series using stainless steel coils, which were immersed in a water bath (Thermomix BU, B Braun Melsungen, Germany, temperature accuracy ±0.3 °C). The equipment was fitted with 10 in-line thermocouples, which were inserted in the liquid stream, to monitor the temperature at the entry and exit of all four PEF treatment chambers and two other strategic points to assist in the control of process temperature. Further details of the fluid handling, temperature control, and recording systems have been described previously (*20*, *21*).

The PEF treatment of the blank SMUF and LF solutions with different electrical conductivities was carried out at an electric field intensity of 35 kV cm^{-1}, with a square pulse width of 2 μs, and at a bipolar pulse frequency of 100 Hz. The flow rate of the sample stream for all experiments was maintained at 60 mL min^{-1}, which allowed a total pulsing time of 19.2 μs and a specific energy input of 40.6−245 kJ L^{-1} for LF samples in 0.2−2 strength SMUF. The maximum temperature of the sample stream did not exceed 30 °C at any of the in-line thermocouples along the PEF processing line. This was achieved by altering the inlet temperature of the samples to compensate for differing levels of PEF-induced heating due to the different electrical conductivities of the various treatment media.

Sample Analysis. Ultrafiltration to determine the free iron concentration in the treated lactoferrin solutions was conducted both within 5 h and after storage of the sample at 4 °C for 5 days after PEF treatment. All other tests except circular dichroism were performed no more than 24 h following PEF treatment. Circular dichroism experiments were conducted within 3 days after the PEF treatment, due to the time required to complete buffer exchange by dialysis.

Determination of LF Content. The concentration of LF was determined using both a spectrophotometric and an enzyme-linked immunosorbent assay (ELISA) method. The LF content determined using the spectrophotometric method (*22*, *23*) is referred to as LF_A throughout this paper. LF preparations were centrifuged at 18000*g* for 15 min at 4 °C. The absorbance of the supernatant at 280 nm was then measured using a spectrophotometer (Spectra Max plus 384, Molecular Devices, Sunnyvale, CA). Untreated LF solutions at 0, 0.1, 0.2, 0.3, and 0.4 mg mL^{-1} in 0.2 strength SMUF were used to establish a standard curve. The ELISA procedure was conducted using a bovine lactoferrin ELISA quantitation kit (catalog no. E10-126, Bethyl Laboratories, Inc., Montgomery, TX) and following the manufacturer's protocol. The concentration of LF samples determined using the ELISA method is referred to as LF_E. All samples were measured in triplicate for both LF_A and LF_E.

Determination of Surface Hydrophobicity. The value of surface hydrophobicity (S_0) was determined by the fluorescent probe 8-anilino-1-naphthalenesulfonic acid hemimagnesium salt hydrate (ANS, 10419, Fluka Sigma, St. Louis, MO), according to the method described by Alizadeh-Pasdar and Li-Chan (*24*). LF samples were diluted to concentrations of 0.05−0.25 mg mL^{-1}. Twenty-five microliters of ANS stock solution (8 mM ANS in 0.1 M phosphate buffer, pH 7.4) were added to 5 mL of LF preparation at each concentration, and the mixture was then stored in the dark at room temperature for 15 min. The fluorescence intensity (FI) of samples at each concentration was measured using a fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan; excitation wavelength, 380 nm; emission wavelength, 480 nm; slit width, 10 nm). The inner filter effect was corrected according to the method proposed by Kubista et al. (*25*). The net corrected FI value was calculated by subtracting the corrected FI value of the preparation without ANS from that of the preparation with ANS. A linear plot was established by plotting the net corrected FI value against the protein concentration; the slope of this plot was used as the index (S_0, arbitrary unit) of surface hydrophobicity. Duplicate measurements were conducted with all samples.

Electrophoresis. Nonreducing sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was conducted using precast gels (NuPAGE Novex 4−12% Bis-Tris Gel, NP3029BOX), running buffer [2-(*N*-morpholino)ethane sulfonic acid (MES) SDS Running Buffer,

NP0002], and other reagents supplied by Invitrogen Life Technologies (Carlsbad, CA) and following the manufacturer's protocols.

Far-UV Circular Dichroism (CD). Far-UV CD was employed to examine secondary structure of LF (*26*). Untreated or PEF-treated LF solutions were dialyzed first against deionized water (4 °C, 16 h) and then against 10 mM phosphate buffer (pH 7.0, 4 °C, 24 h), using a regenerated cellulose membrane (Spectra/Por Dialysis Membrane 7, molecular weight cut off 1000 Da, catalog no. 132104, Spectrum Laboratories Inc., Rancho Dominguez, CA). Subsequently, the dialyzed LF solutions were diluted (3:1) with 10 mM phosphate buffer (pH 7.0), and the protein concentration (LF_A) was determined as described above. CD spectra of the diluted LF solutions were obtained at 25 °C from 260 to 190 nm with a spectral resolution of 0.2 nm using a Jasco J-815 spectropolarimeter (Jasco Corp., Tokyo, Japan). CD spectra were recorded using a 0.1 cm path length quartz cell at a speed of 100 nm min^{-1}, a response time of 0.125 s, and a bandwidth of 1 nm (*27*). Reported spectra are an average of five scans and normalized to mean residue ellipticity θ_{mrw} (deg cm^2 $dmol^{-1}$) using eq 1 (*26*)

$$[\theta]_{mrw,\lambda} = \frac{MRW \times \theta_\lambda}{10 \times d \times c} \quad (1)$$

where MRW is the mean residual weight of LF (1138 g mol^{-1}), θ_λ the observed ellipticity (degrees) at wavelength λ, d the path length (=0.1 cm), and c the protein concentration (g mL^{-1}). The percentage of α-helix, β-sheet, and random coil was analyzed using the CDSSTR method and database SP175 (*28*) via the DicrobWeb online service proposed by Whitemore and Wallace (*29*).

Intrinsic Fluorescence Spectroscopy. The tryptophan emission fluorescence spectra were recorded to indicate the tryptophan environment, which is related to changes in tertiary structure of LF (*27*). Excitation of the 0.4 mg mL^{-1} LF solutions was performed at 274 nm, and emission spectra were obtained from 300 to 400 nm using a fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan) with a band slit of 10 nm, a resolution of 1 nm, and a scanning rate of 60 nm min^{-1}. All measurements were performed in triplicate.

Determination of Free Iron Concentrations in LF Solutions. The effect of PEF treatment on the iron-binding affinity of LF was investigated by determination of the release of ferric ion from the LF molecules into the aqueous phase. The free ferric ions were separated from those bound to LF molecules using ultrafiltration. LF samples (14 mL) were centrifuged at 5000*g* and 4 °C for 10 min to separate the protein aggregates from the supernatant. The supernatant (12 mL) was then transferred to a centrifugal ultrafiltration device with a nominal molecular weight limit (NMWL) of 3000 Da (Amicon Ultra-15 3K, UFC900308, Millipore, Bedford, MA) and centrifuged at 4000*g* and 4 °C for 40 min to remove the protein molecules. The iron concentration of the filtrate was determined using an inductively coupled plasma−optical emission spectrometer (ICP-OES, 730-ES, Varian Inc., Palo Alto, CA) and expressed as $C_{control}^{aqueous}$ for untreated LF control samples or $C_{PEF}^{aqueous}$ for PEF-treated LF samples, respectively. LF samples that were not subjected to the ultrafiltration process were also analyzed before PEF treatment to determine the total iron concentration in the preparation ($C_{control}^{LF+aqueous}$). In addition, the iron concentration in the SMUF of different strengths was determined before ($C_{control}^{SMUF}$) and after (C_{PEF}^{SMUF}) PEF treatment to detect the potential release of iron from the electrodes in the treatment chambers. The ICP-OES analyses were conducted in duplicate by Agrifood Technology (Werribee, VIC, Australia). The concentration of iron remaining bound to LF before ($C_{control}^{LF}$) and after PEF treatment (C_{PEF}^{LF}) was calculated using eqs 2 and 3, respectively:

$$C_{control}^{LF} = C_{control}^{LF+aqueous} - C_{control}^{aqueous} \quad (2)$$

$$C_{PEF}^{LF} = C_{control}^{LF+aqueous} - (C_{PEF}^{aqueous} - C_{control}^{aqueous}) - C_{PEF}^{SMUF} \quad (3)$$

Statistical Analysis. Statistical analysis was performed by applying two-way analysis of variance (ANOVA) for PEF using SMUF strength as the block for calculation of the least significant difference (LSD) at the confidence level of 95%. Analysis of data was carried out using GenStat software (version 11, VSN International Ltd., Hemel Hempstead, U.K.), and the difference between the mean values greater than the $LSD_{0.95}$ was determined as significant.

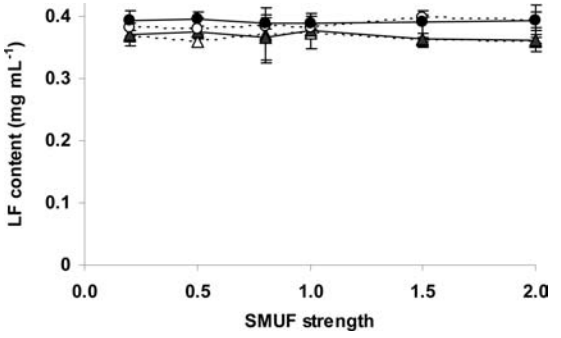

Figure 2. Content of untreated (○, △) or PEF-treated (35 kV cm^{-1}, 19.2 μs, ●,▲) LF samples (0.4 mg mL^{-1} in 0.2−2 strength SMUF) determined using spectrophotometric (LF_A, ○, ●; $LSD_{0.95}$ = 0.015) and ELISA (LF_E, △, ▲; $LSD_{0.95}$ = 0.013) method.

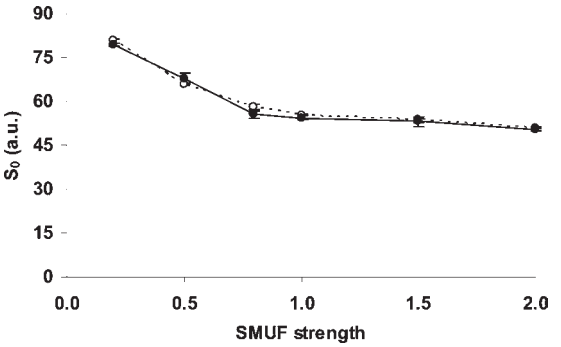

Figure 3. Surface hydrophobicity of untreated (○) or PEF-treated (35 kV cm^{-1}, 19.2 μs, ●) LF samples (0.4 mg mL^{-1} in 0.2−2 strength SMUF). $LSD_{0.95}$ = 3.4.

RESULTS AND DISCUSSION

Effect of PEF Treatment on LF Content. The spectrophotometric method (LF_A) detects the decrease in protein concentration due to aggregation/precipitation, whereas the ELISA method (LF_E) determines the concentration of LF that is sufficiently structurally intact to be detected by the specific antibody. For the LF samples in all strengths of SMUF, PEF treatments did not change ($p > 0.05$) the LF content of either LF_A or LF_E (**Figure 2**). It has been reported that PEF treatment at a similar electric intensity (36.7 kV cm^{-1}) but with much longer times (50−200 exponential pulses with a pulse width of 2 μs) did not change the concentration of LF (determined using specific antibody) in diluted raw skim milk and diluted whey with similar electrical conductivity (0.2 S m^{-1} for both media) to the 0.2 strength SMUF (0.17 S m^{-1}) used in the current study (*14*). This suggests that neither long treatment time (up to 400 μs) nor high electrical conductivity of treatment medium (up to 1 S m^{-1}) had an effect on the concentration of specifically detectable LF when the PEF treatment was conducted at about 35 kV cm^{-1} and 30 °C.

Effect of PEF Treatment on LF Surface Hydrophobicity. The surface hydrophobicity value of the untreated LF control substantially decreased ($p < 0.05$) from 80.5 in 0.2 strength to 57.8 in 0.8 strength SMUF. The further increase in the strength of SMUF from 1 to 2 resulted in only a minor decrease in S_0 from 54.9 to 51 (**Figure 3**). This decreasing trend in LF surface hydrophobicity may be explained by the anionic nature of the fluorescent probe ANS. The greater amount of cations in the SMUF of higher strengths may interact with ANS (*24*), thereby influencing the measurement of the surface hydrophobicity of LF, which was based on the binding of ANS to LF molecules.

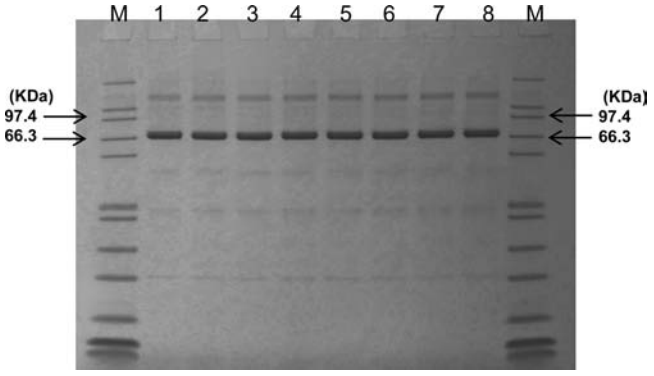

Figure 4. Electrophoretic mobility of untreated (lanes 1, 3, 5, and 7) and PEF-treated (lanes 2, 4, 6, and 8; 35 kV cm^{-1}, 19.2 μs) LF (0.4 mg mL^{-1}) in 0.2, 1, 1.5, and 2 strength SMUF, respectively.

However, when compared with the respective untreated controls at each of the SMUF strengths, PEF treatment did not change ($p > 0.05$) the S_0 (**Figure 3**), indicating that PEF treatment did not cause unfolding of LF molecules at any of the ionic strengths tested.

Effect of PEF Treatment on Electrophoretic Mobility of LF. PEF treatment did not cause any change in the electrophoretic mobility of LF at any of the SMUF strengths tested (**Figure 4**). The identical gel patterns between the PEF-treated LF and untreated controls suggest that PEF treatment did not cause aggregation of LF in any of the media tested (*30*, *31*).

Effect of PEF Treatment on the Secondary and Tertiary Structures of LF. Far-UV CD spectroscopy was used to detect possible changes in the secondary structure of LF molecules. The spectra show no difference between the untreated and PEF-treated LF samples in either 0.2, 1, or 2 strength SMUF (**Figure 5**, panels **a**, **b**, and **c**, respectively). The proportions of α-helix (15−17%), β-sheet (32−33%), turn (12%), and random coil (38−39%) were not changed ($p > 0.05$) in any of the PEF-treated LF samples. These results suggested that PEF treatments did not alter the secondary structure of LF molecules.

PEF treatments did not affect the tryptophan emission spectra of the LF in either 0.2, 1, or 2 strength SMUF, compared to the corresponding untreated control (**Figure 5**, panels **d**, **e**, and **f**, respectively), indicating that PEF treatments did not change the local environment around tryptophan residues on LF. These results suggest that PEF treatment has not changed this part of the tertiary structure of LF molecules. However, other parts of the tertiary structure still may be altered by PEF treatment, but, if any, these potential changes are likely to be subtle. The specific anti-LF antibody used in the current study cannot detect the LF with a substantial conformational change (*32*), which should be reflected by a decreased LF_E concentration. However, the LF_E concentration determined in this study showed no decrease after PEF treatment.

Effect of PEF Treatment on the Ferric Ion Release from LF Molecule. On the basis of the molar masses of iron (56 g mol^{-1}) and LF (78×10^3 g mol^{-1}) and the total iron content ($C_{control}^{LF+aqueous}$, 174 μg L^{-1}) of the 0.4 mg mL^{-1} LF solution, the molar ratio of LF-bound iron to LF (Fe/LF) in the untreated sample was calculated as approximately 0.61. This is consistent with an iron saturation level of 24.5%, as reported in the sample specification, considering that each LF molecule has two iron-binding sites. **Figure 6** shows the changes in the iron content of the SMUF solutions ($C_{control}^{SMUF}$, C_{PEF}^{SMUF}) and in the aqueous phase of the LF preparations ($C_{PEF}^{aqueous}$, $C_{control}^{aqueous}$), as determined using ICP-OES. The iron content increased slightly ($p < 0.05$) with the increase of the SMUF strength; this could be from trace amounts of iron in lactose and chemicals used to prepare SMUF. PEF treatment did not cause the release of measurable amounts of iron from the electrodes into the sample because the iron content remained constant ($p > 0.05$) in all PEF-treated SMUF solutions. For the LF sample in 0.2 strength SMUF, little or no ferric ion was released into the aqueous phase after PEF treatment ($p > 0.05$). However, when the treatment was conducted with the LF sample in 0.5 strength SMUF, the concentration of free iron in the aqueous phase ($C_{PEF}^{aqueous}$) increased to 18 μg L^{-1}. The LF samples in higher ionic strength environments released more ferric ions after the PEF treatment, with ($C_{PEF}^{aqueous}$ being 51−120 μg L^{-1} for the LF treated in 0.8−2 strength SMUF (**Figure 6**). Accordingly, the concentrations of iron remaining bound to LF after PEF treatment ($C_{PEF}^{aqueous}$) were calculated as 174, 160, 132, 119, 104, and 80 μg L^{-1} for the LF samples treated in 0.2, 0.5, 0.8, 1, 1.5, and 2 strength SMUF, respectively. To study the correlation between the electrical conductivity of PEF treatment medium and the iron release, the natural logarithm of the ratio of C_{PEF}^{LF} to $C_{control}^{LF}$ [$\ln(C_{PEF}^{LF}/C_{conrtol}^{LF})$] was plotted against the electrical conductivity of the treatment medium (**Figure 7**). The data well fitted a first-order reaction model ($R^2 = 0.9819$). The increase in the electrical conductivity of the treatment medium led to a marked increase in the release of ferric ions from the LF molecules during PEF treatments, following first-order kinetics. This means that the degree of iron depletion from LF can be controlled by adjusting the ionic strength of the PEF medium. It is noted that although all experiments in this study were carried out at the same temperature, electric field intensity, and total treatment time, the specific pulse energy delivered to the LF samples varied. The pulse energy to the samples in 2 strength SMUF was about 6 times higher than that delivered to the samples in 0.2 strength SMUF. Changes in the specific energy input might be achieved by increasing the total treatment time but keeping the electric field intensity and medium electrical conductivity constant. It is acknowledged that the changes in conductivity and specific energy may also influence other parameters such as the shape and width of the electric pulse or the distribution of the electric field. However, it is not yet possible to identify and quantify any concurrent effects of sample conductivity on pulse shape and field intensity distribution. Further research would be required to investigate these more complex effects.

In the literature, the reported PEF-induced improvements in protein functionality (such as the better emulsifying capacity and stability of liquid egg white after PEF treatment at 30 kV cm^{-1} and a long time of 800 μs) have been proposed to be associated with the PEF-induced unfolding of egg white proteins (*13*). In the study reported here, the results indicate that PEF treatment, even at the highest electrical conductivity tested, appeared not to cause permanent changes to the physicochemical properties of LF, including LF content, surface hydrophobicity, and electrophoretic mobility. In addition, changes in the secondary structure of LF molecules could not be detected after PEF treatment. The iron-binding sites of LF are close to some sections of random coils (**Figure 1**), so the release of iron is unlikely to cause major changes in the secondary structure of LF. It is reasonable to assume that the release of iron from LF molecule could be associated with subtle changes in the tertiary structure of the PEF-treated LF. However, the unchanged environment around tryptophan suggests that PEF did not change this part of LF tertiary structure. It is acknowledged that because of the unavoidable time delays between the PEF treatment and the analytical procedures, it is possible that there may be some transient changes in tertiary structure which result in the release of ferric ion from LF molecules during the treatment, but the conformation of LF

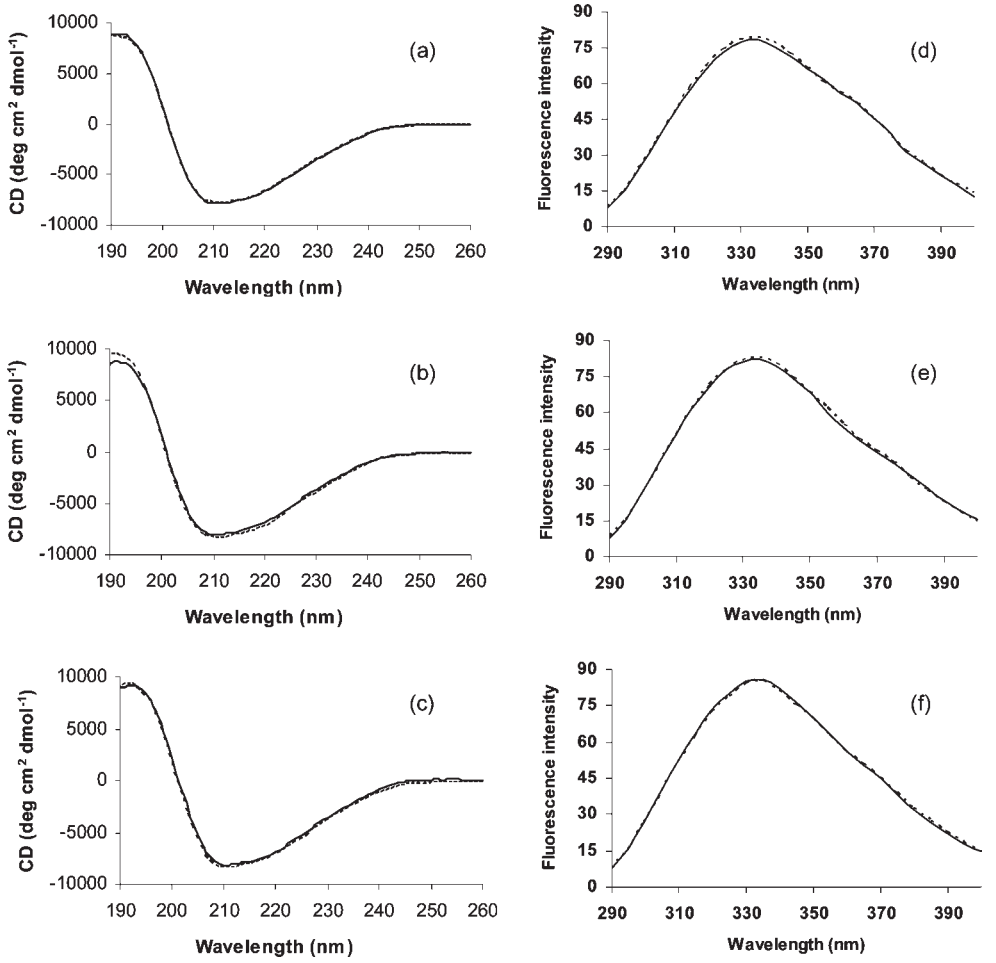

Figure 5. Far-UV CD (**a**–**c**) and tryptophan emission (**d**–**f**) spectra of 0.4 mg mL^{-1} LF in 0.2 (**a**, **c**), 1 (**b**, **d**), and 2 (**e**, **f**) strength SMUF before (- - -) and after (—) PEF treatment at 35 kV cm^{-1} for 19.2 μs.

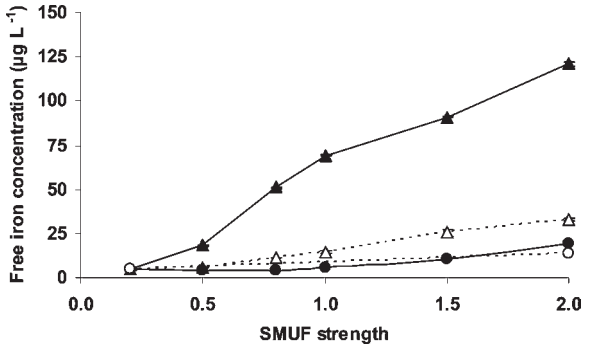

Figure 6. Concentration of free iron in blank 0.2–2 strength SMUF ($C^{SMUF}_{control}$, ○; C^{SMUF}_{PEF}, ●; $LSD_{0.95}$ = 0.5) and LF samples (0.4 mg mL^{-1} in 0.2–2 strength SMUF; $C^{aqueous}_{control}$, △; $C^{aqueous}_{PEF}$, ▲; $LSD_{0.95}$ = 2.5) before (○, △) and after (●, ▲) PEF treatment (35 kV cm^{-1}, 19.2 μs).

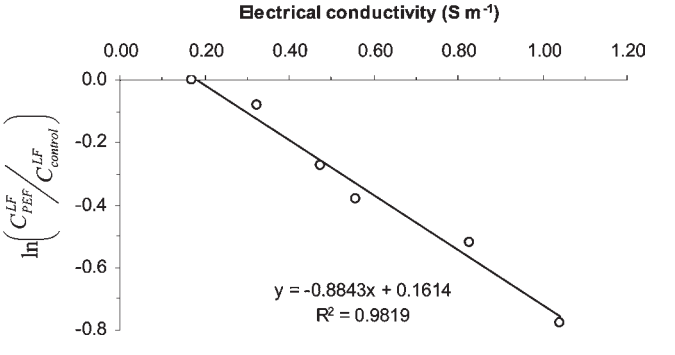

Figure 7. Correlation between the electrical conductivity of treatment medium and the iron release from LF (expressed as the ratio of the concentration of iron bound to the PEF-treated LF to that of the control, $\ln(C^{LF}_{PEF}/C^{LF}_{conrtol})$ after PEF treatment (35 kV cm^{-1} for 19.2 μs) of 0.4 mg mL^{-1} LF in various strength SMUF (0.2–2 strength, electrical conductivity = 0.17–1.04 S m^{-1}).

returned to its native status. Further studies are required to fully understand the mechanism of PEF-induced iron depletion of LF molecules.

The iron-depleted LF (Apo-LF) has been found to have a greater antimicrobial effect against some strains and species than LF with natural levels of iron (N-LF) or LF saturated with iron (Holo-LF), including the inhibition of growth of *Aspergillus fumigatus* conidia (*33*) and a bacteriostatic effect against enteropathogenic *Escherichia coli* (*34*). In addition, Chan and Li-Chan (*35*) reported that the digestion of Apo-LF and N-LF by pepsin, in addition to the main product lactoferricin (which has a stronger antimicrobial effect than undigested LF), generated other low molecular weight cationic peptides with potential bioactive properties. The authors found that iron saturation level had no effect on the amount of lactoferricin produced. However, compared with the N-LF, the digestion of Apo-LF produced additional low molecular weight cationic peptides.

Traditionally, the iron depletion of LF has been achieved using chemical methods, such as lowering the pH to 2 or adding strong

cation chelating agents (e.g., EDTA) (*1*, *31*, *36*). An improvement on the method to deplete iron from LF molecules would favor the growing research interest in Apo-LF. The results from this study suggest that PEF treatment has the potential to be a physical method to produce Apo-LF without appreciably changing the conformation of the LF molecules. In addition, it is difficult to control the degree of iron depletion when using the traditional methods, but PEF treatment may provide the possibility to produce LF with different iron depletion levels by adjusting the ionic strength or electrical conductivity of the process medium. In addition, the bioactive properties of the Apo-LF generated by PEF also need to be evaluated in comparison to that produced by the traditional methods.

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