Effects of Pulsed Electric Fields on Structural Modification and Rheological Properties for Selected Food Proteins

by

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ABSTRACT

The application of pulsed electric fields (PEF) in food processing is a novel technique that requires careful study. An improved understanding of the effects of PEF treatments on structural modification, surface hydrophobicity, thermal properties and rheological properties of food proteins is needed for the application of this method to the improvement of functional properties of food proteins in food systems. Two main PEF parameters were studied: electric field intensity ranging from 12 to 25 kV cm^{-1}, and number of pulses ranging from 10 to 120. PEF effects on the structural modification, surface hydrophobicity, thermal properties and rheological properties for selected food proteins were investigated using fluorescence spectroscopy and differential scanning calorimetry (DSC) along with rheological measurements. Whey protein isolate (WPI), soy protein isolate (SPI), soy milk and reconstituted skim milk were used in this study. The structural modification, surface hydrophobicity and thermal properties of WPI and SPI all changed with PEF treatments. The enthalpy of whey protein isolate decreased with PEF treatments. The apparent enthalpy and denaturation temperatures of WPI samples were modified by PEF treatments. The remaining native whey protein was about 56% by a PEF treatment of 20 kV cm^{-1} and 30 pulses. The intrinsic tryptophan fluorescence intensity and the extrinsic fluorescence intensity of whey protein and soy protein all increased with PEF treatments. The intrinsic tryptophan fluorescence of whey protein and soy protein appeared as a red shift of 2-6 nm. The extrinsic fluorescence of whey protein and soy protein appeared as a blue shift of 2-6 nm with PEF treatments. The surface hydrophobicity of whey protein and soy protein increased with PEF treatments. PEF treatments with different electric field intensities and number of pulses affected the rheological properties and color properties of reconstituted skim milk and soy milk. The apparent viscosity and shear stress of soy milk and skim milk increased with PEF treatments. For skim milk, the apparent viscosity and consistency index increased with increasing electric field intensity and number of pulses. The apparent viscosity increased from 2.12 to 4.25 (10^{-3} Pa.s) for the untreated control and 60 pulses of 20 kV cm^{-1}. The consistency index increased from 1.42 to 1.54 (10^{-3} $Pa.s^n$) for the control and PEF-treated

at 60 pulses and 20 kV cm^{-1}. For soy milk, the apparent viscosity of soy milk increased from 6.62 to 7.46 (10^{-3} Pa.s) by increasing electric field intensity from 18 to 22 kV cm^{-1} and number of pulses increasing from 0 (the no treated control) to 100. This study confirmed that electric field intensity and number of pulses affected the structural modification, surface hydrophobicity, thermal and rheological properties for selected food proteins.

RÉSUMÉ

L'utilisation de champs électriques pulsés (CEP) dans la transformation d'aliments est une technique de pointe qui nécessite des études approfondies. Une meilleure compréhension de l'effet de traitements CEP sur les modifications structurales, l'hydrophobicité en surface et les propriétés rhéologiques de protéines alimentaires est nécessaire à l'application de cette méthode pour l'amélioration des propriétés fonctionnelles de protéines alimentaires dans les systèmes alimentaires. Deux principaux paramètres CEP furent étudiés: l'intensité du champ électrique, variant de 12 à 25 kV cm^{-1}, et le nombre de pulsions, variant de 10 to 120. L'effet de traitements CEP sur les modifications structurales, l'hydrophobicité en surface et les propriétés rhéologiques de certaines protéines alimentaires furent étudiés par spectroscopie fluorescente et analyse calorimétrique à compensation de puissance (ACCP) et diverses techniques rhéologiques Un isolat de protéine lactosérique (IPL), un isolat de protéine de fève soja (IPFS), du lait de soja et du lait écrémé reconstitué servirent dans cette étude. La modification structurale, l'hydrophobicité en surface et les propriétés thermiques de l'IPL et de l'IPFS furent modifiées par les traitements CEP. L'enthalpie Un isolat de protéine lactosérique (IPL) a diminué avec les traitements CEP. L'enthalpie apparente et les températures de dénaturations des échantillons de IPL ont été modifié après les traitements. La lactalbumine indigène restante était environ de 56% après un traitement de PEF de 20 kV cm^{-1} et 30 pulsions. Ces traitements augmentèrent à la fois la fluorescence intrinsèque du tryptophane et la fluorescence extrinsèque des protéines lactosériques et de soja. La fluorescence intrinsèque du tryptophane de l'IPL et de l'IPFS montra un décalage de 2-6 nm vers le rouge après le traitement CEP. De même l'hydrophobicité en surface des protéines lactosériques et de soja augmentèrent après le traitement CEP. L'intensité et le nombre de pulsion du champ électrique influencèrent tous deux les propriétés rhéologiques et la coloration du lait écrémé reconstitué et du lait de soja. La viscosité apparente et la contrainte cisaillement du lait écrémé et du lait de soja augmentèrent après les traitements CEP. Pour le lait écrémé, la viscosité apparente et l'index de consistence ont augmenté avec l'augmentation de l'intensité du champs électrique et le nombre de

l’impulsions. La viscosité apparente a augmenté de 2.12 à 4.25 (10^{-3} Pa.s) pour les échantillons prises comme control et de 60 pulsions de 20 kV cm^{-1}. L’index de consistance a augmenté de 1.42 à 1.54 (10^{-3} $Pa.s^{n}$) pour le control et 60 pulsions de 20 kV cm^{-1}. Pour le lait de soja, la viscosité apparente a augmenté de 6.62 à 7.46 (10^{-3} Pa.s) en augmentant les champs électriques de 18 à 22 kV cm^{-1} et le nombre des pulsions a augmenté de 0 (pour le control) à 100. Cette étude confirma que l'intensité du champ électrique et le nombre de pulsions eurent une importante influence sur les modifications structurales, l'hydrophobicité en surface ainsi que les propriétés thermales et rhéologiques des protéines alimentaires choisies.

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CONTRIBUTION OF AUTHORS

The role and contribution made by different authors are as follows: The principal author is Mr. Bob Yongsheng Xiang. He is the Ph.D candidate who planned, designed, and executed all the experiments, completed the data analysis, and wrote the manuscript for scientific publication. Dr. Michael O. Ngadi is the thesis supervisor, who guided the candidate in planning, design, and execution of the experiments and analysis of the data during the course of the entire program. He corrected, edited, and reviewed all the manuscripts sent for publication. Dr. Benjamin Simpson also contributed in planning and execution some aspects of the project. He allowed the candidate to use his laboratory and contributed in editing manuscripts for scientific publication. Dr. Luz Aracel Ochoa-Martinez, Chemistry and Biochemistry Department of Technical Institute of Durango, Mexico, she worked with the candidate and helped the candidate do some experiments and she also edited one manuscript for scientific publication. Dr. Tanya Gachovska designed PEF generator and the candidate could use this equipment to finish this project and she also helped to editing manuscripts for scientific publication and conference papers. Dr. Marian Simpson helped me to edit some manuscripts for scientific publication and conference papers.

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ABBREVIATIONS AND NOMENCLATURE

Symbol	Definition	Units
ANOVA	analyses of variance	
ANS	1-anilino-8-naphtalene sulfonic acid)	
BSA	bovine serum albumin	
DSC	differential scanning calorimetry	
EFI	extrinsic fluorescence intensity	
FI	fluorescence intensity	
FS	fluorescence spectroscopy	
FTIR	Fourier transform infrared spectroscopy	
IFI	intrinsic fluorescence intensity	
HCl	hydrochloric acid	
HP	high pressure	
O/W	oil-in-water	
PAGE	polyacrylamide gel electrophoresis	
PEF	pulsed electric field	
SMFU	simulated milk ultra filtrate	
SEM	scanning electron microscopy	
SPI	soy protein isolate	
UV	ultraviolet spectroscopy	
W/O	water-in-oil	
WHC	water holding capacity	

WPC	Whey protein concentrate	
WPI	whey protein isolate	
α-LA	α-lactalbumin	
β-LG	β-lactoglobulin	
σ	electric conductivity	S m^{-1}
τ	Pulse width	μs
τ_o	yield stress	Pa
τ	shear stress	Pa
ΔH	apparent enthalpy	J g^{-1}
A	electrode surface area	m^2
a^*	red/greenness	-
b^*	yellowness/blueness	-
C	capacitance	F
C	protein concentration of protein solution	%
C_p	specific heat	J g^{-1} K^{-1}
d	gap between two electrodes	cm
E	electric field intensity	kV cm^{-1}
h	distance between the spheres of the discharger	cm
I	intrinsic fluorescence intensity	a.u.
K	consistency coefficient	$Pa.s^n$
L^*	lightness of colour	-

n	number of pulses	n
n	flow behavior index	dimensionless
P	number of pulses	n
Q	energy input in one capacitor	kJ
R	electrical resistance	Ω
T_d	denaturation temperature	^{o}C
t	treatment time	s
V	voltage	kV
V_o	the break voltage	kV
v	the apparent viscosity	Pa.s

1. INTRODUCTION

1.1 GENERAL INTRODUCTION

Traditional thermal processing methods may cause detrimental changes of food constituents, such as proteins and vitamins, with consequent possible changes in sensory and nutritional characteristics. Non-thermal processing methods are getting more and more attention in the food industry, which recognize their potential to replace or complement traditional thermal processing methods. Pulsed electric fields (PEF) technology has been presented as advantageous in comparison to thermal processing methods because it kills microorganisms while maintaining the original color, flavor, texture, and nutritional value of the foods to which it is applied. PEF technology involves the application of pulses of high voltage to liquid or semi-solid foods placed between two electrodes. Most PEF studies have focused on PEF treatments effects on the microbial inactivation in milk, milk products, egg products, juice and other liquid foods (Qin et al., 1995; Martin-Belloso et al., 1997) and a lesser amount of information is available about the effect of PEF on food constituents, such as food proteins, enzymes, and the overall quality and acceptability of PEF-treated foods. The level of microbial inactivation and enzyme inactivation achieved with PEF treatments depends mainly on the electric field intensity, number of pulses, pulse width, treatment time, pulse wave form, as well as other parameters (Barsotti & Cheftel, 1999). For example, a greater electric field intensity (30-70 kV cm^{-1}) resulted in a greater microbial inactivation (6-9 log reduction) in simulated milk ultrafiltrate (SMUF) and ultra high temperature (UHT) sterilized milk (Zhang et al., 1995a; Qin et al., 1998). Also, the combination of PEF treatment and mild heat treatment (50 to 63°C) demonstrated higher microbial inactivation in pasteurized whole milk than either alone (Dunn & Pearlman, 1987; Reina et al., 1998). PEF treatment is becoming a promising method for milk pasteurization in terms of quality improvement.

At present, it is well demonstrated that PEF of adequate electric field intensity efficiently inactivates many kinds of microorganisms and enzymes (Wouters & Smelt, 1997; Barsotti & Cheftel, 1999; Wouters et al., 1999), but much less is known concerning

PEF effects, in terms of structural modification of proteins, enzymes and other food constituents (Barsotti & Cheftel, 1999; Fernandez-Diaz et al., 2000; Yeom & Zhang, 2001; Perez & Pilosof, 2004; Li et al., 2005). Studies on enzyme inactivation in enzyme solutions or liquid foods by PEF treatments reveal varying results, perhaps partially due to differences in the PEF systems and pulse wave form used (Vega-Mercado et al., 1995; Barbosa-Cánovas et al., 1998; Barsotti & Cheftel, 1999, Yang et al., 2004).

Physical methods (heat or mild alkali treatment), chemical methods (acylation, phosphorylation and deamidation), enzymatic methods (proteases) and high pressure methods (Nir et al., 1994; Qi et al., 1997; Horiuchi et al., 1978; Liu et al., 2005; Bouaouina et al., 2006) have all been used to modify functional properties of food proteins, but PEF offers a technique by which the functional characteristics of food proteins can be modified in a consumer friendly, "green" manner leading to food products of added value. Very limited information is available on the effect of PEF on the structural and functional properties of whey and soy proteins. To our knowledge, only Perez & Pilosof (2004) and Li et al. (2007) have demonstrated that under certain PEF conditions (high pulse width and high electric field intensity) led to protein structural modification and improved the rheological and functional properties of β-lactoglobulin and soy proteins. Further experimental work is required to understand the structural modification or interactions taking place under PEF treatments of some food proteins and to make structural modification and improve the desired functional properties of food proteins.

A number of thermodynamic studies have revealed that the native states of globular proteins are only marginally stable relative to their modified states. Structural characterization of the non-native (partially unfolded and modified) states is essential for understanding the mechanisms of protein unfolding and the principle of structure stabilization (Kim & Baldwin, 1990; Dill & Shortle, 1991). The study of protein modification and rheological properties of food proteins as a function of PEF treatments can provide unique information on unfolding mechanisms and rheological properties of food proteins. But such information is almost nil; in addition, very little work has been done on the interactive effects of other factors that affect structural modification and

rheological properties of food proteins. Consequently, the main objectives of this study were to generate information regarding protein modification and rheological properties under PEF treatments.

The overall objectives of this study were to develop new PEF methods to make the structural modification and increase surface hydrophobicity of food proteins, obtain the desirable functional properties for food proteins and increase the utilization of food proteins in food industry.

1.2 OBJECTIVES

The specific objectives of this study are as follows:

1. To investigate the effects of PEF on the structural modification and surface hydrophobicity of whey protein isolate (WPI) under different PEF conditions (different electric field intensities and pulses) and to confirm the results by using a fluorescence spectroscopy technique.
2. To investigate the structural modification and thermal properties of whey protein isolate (WPI) with PEF treatments and to confirm that PEF treatments make structural modification and thermal properties changes of WPI by using fluorescence spectroscopy and differential scanning calorimetry (DSC) techniques.
3. To investigate the structural modification and surface hydrophobicity of soy protein isolate (SPI) with PEF treatments (different electric field intensities and pulses) and to confirm the results by using fluorescence spectroscopy technique.
4. To investigate the effects of PEF treatments (different electric field intensities and pulses) on the flow behavior and viscosity of reconstituted skim milk using a rheometer.
5. To investigate the effects of PEF treatments on the rheological and color properties of soy milk at different PEF conditions (different electric field

intensities and pulses) and to confirm the results by using a rheometer and spectrophotometer.

2. LITERATURE REVIEW

2.1 PULSED ELECTRIC FIELD PROCESSING

2.1.1 General information

Pulsed electric fields (PEF) technology is a nonthermal processing technology that may have the potential to replace or supplement traditional thermal processing methods (Qiu et al., 1998). The use of PEF technology in foods reduces pathogen levels while increasing food shelf life; retaining flavor, color and nutritional properties of foods and also improving protein functionality (Dunn, 1996). PEF technology aims to offer consumers high-quality foods. For food quality attributes, PEF technology is considered superior to traditional thermal processing methods because it avoids or greatly reduces detrimental changes in the sensory and physical properties of foods (Quass, 1997). For PEF treatments, foods can be treated at roughly ambient temperature or above ambient temperature for only a few microseconds, minimizing the energy loss caused by heating (Barbosa-Cánovas et al., 1999).

2.1.2 PEF system

The PEF processing system is a simple electrical system which consists of a number of components, including a high-voltage power supply, an energy storage capacitor bank, a charging resistor, a switch, treatment chamber(s), voltage, current and temperature probes, a cooling system and a control panel to monitor operations for the whole PEF system. In case of a continuous system, a pump is used to conduct liquid food through the treatment chamber(s). The cooling system is normally used to diminish the ohmic heating effect and maintain the sample temperature in the treatment chamber.

In PEF technology, the energy derived from a high voltage power supply is stored in one or several capacitors and discharged through a food material to generate the necessary electric field in the food (Barbosa-Cánovas et al., 1999). The capacitance (C, [F]) of the energy storage capacitor is given by the following equation:

$$C = \tau/R = \tau\sigma A/d \quad (1)$$

Where C is the capacitance of the energy storage capacitor (F), τ is the pulse width (second), R is the electrical resistance (Ω), A is the area of electrode surface (m^2); σ is the electrical conductivity of the food (S m^{-1}); d is the gap between the electrodes (m).

The energy stored in one capacitor (Q [J m^{-3}]) is given by the following equation:

$$Q = \tfrac{1}{2} C V^2 \quad (2)$$

Where, C is the capacitance (F), V is the charging voltage (kV).

The energy stored in the capacitors can be discharged almost instantaneously (in a nanosecond) at very high levels of power (Vega-Mercado et al., 1997). The discharge occurs in a treatment chamber in which the food is placed or circulates through a small gap between two electrodes (Barbosa-Cánovas et al., 1999). When a trigger signal is activated, a high-voltage switch is closed and the charge stored in the capacitor flows through the food in the treatment chamber (Zhang et al., 1995a; Barsotti et al., 1999). In order to avoid undesirable thermal effects, cold water is recirculated through the electrodes to dissipate the heat generated by the electric current passing through the foods (Barbosa-Cánovas et al., 1999).

2.1.2.1 High voltage power supply

High voltage pulses are supplied to the PEF system via a high voltage generator at required electric field intensity, pulse waveform and pulse width. In general, the high voltage power supply is used to charge the capacitor bank and store the energy to the capacitor bank. Liquid food may be processed in a static treatment chamber or in a continuous treatment chamber through a pump. For preliminary laboratory-scale studies, the static treatment chamber is used, but a continuous treatment chamber (s) is desirable for the pilot plant or industrial-scale operations. In order to avoid undesirable thermal effects, cold water of the cooling system is recirculated through the electrodes to dissipate the heat generated by the electric current passing through the food (Barbosa-Cánovas et al., 1999).

2.1.2.2 Switch

The switch plays an important role in the efficiency on the PEF system, and it is selected on the basis of its ability to operate at a high voltage and repetition rate. A switch is used to instantaneously discharge energy from the storage capacitor bank across the food held in the treatment chamber.

2.1.2.3 Treatment chamber

The treatment chamber is one of the most important and complex components in the PEF system. At present, there are several different designs developed for this key component, wherein high voltage delivered by the power supply is applied to the product located between a pair of electrodes. The treatment chamber is designed to hold the food between two electrodes during PEF treatments. The treatment chamber can be designed to work in static or continuous modes. In the static mode, food is held between two parallel electrodes, whereas in the continuous mode food circulates between the electrodes in the continuous treatment chamber during PEF treatments.

2.1.3 Factors affecting the outcome of PEF treatments

The effects of PEF on the food system are related to the PEF system and the properties of the liquid food. The most important factors in the PEF system are the electric field intensity, number of pulses, pulse waveform, pulse width, treatment time and treatment temperature. But enzymes and proteins are generally more resistant to electric field intensity and pulses than microorganisms. This requires further investigation, especially on the effects of pH, temperature, resistivity and composition of the enzyme or protein-containing medium or food system (Barsotti & Cheftel, 1999).

2.1.3.1 Electric field intensity

Electric field intensity is one of the main factors that influence microbial inactivation and enzyme inactivation (Dunne et al., 1996). Microbial inactivation increases with an increase in the electric field intensity, above a critical transmembrane potential (Qin et al., 1998). This is consistent with the electroporation theory, in which the induced potential difference across the cell membrane is proportional to the applied electric field intensity. Experiments by Perez & Pilosof (2004) have indicated that millisecond-order pulses at an electric field intensity of 12.5 kV cm^{-1} partially modified the protein structure of β-lactoglobulin concentrate and egg white. Effects of PEF on enzymes or other proteins appear to depend on the properties of proteins, on the characteristics of the PEF system used, and on the electric process parameters, especially the electric field intensity and the number of pulses (Barsotti et al., 2002).

For the parallel-plate static electrodes, the electric field intensity is given by

$$E = V/d \tag{3}$$

where E is the electric field intensity (kV cm^{-1}), V is the voltage between two electrodes (kV), and d is the gap between two electrodes (cm).

To achieve microbial inactivation the applied electric field intensity needs to be greater than the critical electric field intensity for a particular microorganism (Castro et al., 1993). It is important that the electric field intensity should be evenly distributed in the treatment chamber to achieve an efficient treatment. Electric field intensities of smaller than 4-8 kV cm^{-1} usually do not affect microbial inactivation (Peleg, 1995). In general, the electric field intensity required to inactivate microorganisms in foods in the range of 12-45 kV cm^{-1}. The fact that microbial inactivation increases with increases in the applied electric field intensity and can be attributed to the high energy supplied to the cell suspension in a liquid product (Pothakamury et al., 1995; Liu et al., 1997; Wouters et al., 1999). Because enzymes and proteins are generally more resistant to electric field intensity and pulses than microorganisms, a greater electric field intensity and higher number of pulses is needed (Barsotti & Cheftel, 1999), most importantly pulses with a large pulse width (Perez & Pilosof, 2004).

2.1.3.2 Pulse wave shapes

Exponential decay and square pulse wave forms are the two most commonly used pulse waveforms (Sepulveda-Ahumada, 2003). Square wave pulses bear more energy and are more lethal than exponential decay waveform pulses since under square pulses, the applied treatment is maintained at a consistent intensity over the entire duration of the pulse. Zhang et al. (1994) reported 60% greater inactivation of *Saccharomyces cerevisiae* when using square pulse waveforms than exponential decay pulse waveforms.

Both pulse waveforms can be applied in a mono or bi-polar fashion. Bi-polar pulses are more lethal than mono-polar pulses because the bi-polar pulses cause alternating changes in the movement of charged molecules which lead to extra stress in the cell membrane and enhance its electric breakdown (Barbosa-Cánovas et al., 1999).

2.1.3.3 PEF operation mode

It has been reported that a continuous treatment chamber caused more microbial inactivation than a batch one (Martín et al., 1997), possibly due to a more uniform electric field. Martín-Belloso et al. (1997) reported that to achieve a 2 log reduction of *E. coli* inoculated in milk by batch mode, 64 pulses and 35 kV cm^{-1} were needed, whereas only 25 pulses and 25 kV cm^{-1} was sufficient in continuous mode.

2.1.3.4 PEF treatment time

PEF treatment time is calculated as the number of pulses multiplied by the pulse width (Barbosa-Cánovas et al., 2000). Sepúlveda (2003) proposed that a PEF treatment time between 0.1 to 0.5 ms produced the best results for microbial inactivation. The pulse width is defined as the time where the peak field is maintained for square wave pulses or the time until decay to 37% for exponential decay pulses. Typically, increasing the number of pulses causes an increase in treatment time, as the pulse width is fixed by the impulse generation setup. Martin-Belloso et al. (1997) found that pulse width influenced

microbial inactivation. A longer pulse width resulted in greater inactivation. However, an increase in pulse width may also result in an undesirable food temperature increase and promote electrolytic reactions and electrodeposition at the electrode surfaces (Zhang et al., 1995b). In general, PEF treatments are applied in the form of short pulses to avoid excessive heating or undesirable electrolytic reactions (Sepúlveda & Barbosa-Cánovas, 2005). But for the enzymes and proteins in foods, the system needs to have wide pulse width to get good results and affect protein structural modification (Perez & Pilosof, 2004).

2.1.3.5 Treatment temperature

PEF treatment temperature is also a very important factor affecting microbial inactivation and protein modification. In general, the lethality of PEF treatments increases with an increase in processing temperature; therefore, a proper cooling device is necessary to maintain temperatures below levels that affect nutritional, sensory or functional properties of food products (Wouters et al., 1999). Application of PEF at mild temperatures (50 to 60°C) has been suggested as a way to enhance the effectiveness of PEF treatments (Dunn & Pearlman, 1987). Treatment temperature has a highly synergetic effect in PEF treatment efficacy, as it has a significant influence on cell membrane fluidity and stability. Barbosa-Cánovas et al. (1999) confirmed that maintaining a moderate temperature (about 50-60°C) treatment could have a synergistic effect on inactivation rate.

2.1.3.6 Food related factors

2.1.3.6.1 Food composition

Food components such as fat and protein have been related to protective effects of microorganisms against PEF, such as the capacity of some substances to shield microorganisms from an applied field, or the ability of some chemical species to stabilize or prevent ion migration.

However, this protective effect is not always the case, when one considers different microorganisms. Reina et al. (1998) compared the effect of PEF in milk with different fat contents. They inoculated *L. monocytogenes* into skim milk, 2% fat milk, and whole milk, and evaluated the effects of the fat content on the inactivation rates: no differences were observed. Therefore, further study is needed to clearly explain these phenomena.

2.1.3.6.2 Electrical conductivity

The electrical conductivity of a medium (σ, S m^{-1}), which is defined as the ability to conduct electric current, is an important variable in PEF treatment. The electrical conductivity is given by:

$$\sigma = d/RA \quad (4)$$

Where σ is the electric conductivity (S m^{-1}), R is the resistance of the liquid food (Ω), A is the electrode surface area (m^2), and d is the gap between two electrodes (cm).

Foods with high electrical conductivities (low resistivity) exhibit smaller electric fields across the treatment chamber and therefore are not ideal for PEF treatments. This is because an increase in electrical conductivity results from increases the ionic strength of a liquid and an increase in the ionic strength of a food results in a decrease in the inactivation rate. Furthermore, an increase in the difference between the electrical conductivity of a medium and the microbial cytoplasm weakens the membrane structure due to an increased flow of ionic substance across the membrane (Jayaram et al., 1992).

2.1.3.6.3 pH and ionic strength

Vega-Mercado et al. (1996) studied the effect of ionic strength of the medium (simulated milk ultra filtrate, SMFU) during PEF treatment. They confirmed that the lower the pH and ionic strength, the greater the inactivation rate. When the ionic strength decreased from 168 to 28 mM, the inactivation rate increased from below detection limits to 2.5 log cycles. Also, when the pH reduced from 6.8 to 5.7, the inactivation rate

increased from 1.5 to 2.2 log cycles. The PEF treatment and ionic strength were responsible for electroporation and compression of the cell membrane, whereas the pH of the medium affected the cytoplasm when the electroporation was complete (Dunne et al., 1996).

2.1.4 PEF effects on proteins in liquid foods

Proteins are very important ingredients in foods, providing important nutritional value and functional properties, as well as providing a desirable textural quality to foods. The nutritional and functional properties of food proteins are primarily determined by protein molecular structure and behavior and their concentration, but also by several other variables. The functional performance of the protein naturally also depends on its environment during the application of PEF: temperature, pH, ionic strength and composition, solvent quality, presence of compounds, including enzymes that can chemically modify proteins (Walstra et al., 2006).

For protein modification and enzyme inactivation, the first step that takes place is normally protein unfolding. This phenomenon is considered as reversible, and consists of a structural modification due to a modification of the balance of forces that maintains the protein native structure. After unfolding, some secondary structures may change. These changes are either covalent changes, which result in chemically modified proteins or noncovalent changes, which may lead to an incorrect folding or to aggregation (Klibanow, 1983; Mañas & Vercet, 2006). Protein structure is very dependent on environmental factors, such as pH, temperature, salinity, or ionic strength, elevated pressure, which can affect the delicate equilibrium that maintains the protein's native structure (Walstra et al., 2006). In the protein's native state, it is held together by a delicate balance of forces: hydrophobic, ionic and van der Wal interactions (Mañas & Vercet, 2006).

How can PEF treatments modify protein structure? It seems reasonable to think that the application of PEF could ionize some chemical groups and also break electrostatic interactions inside a protein's polypeptide chain of protein. Investigations of

PEF effects on protein structure are descriptive and do not indicate the possible mechanisms that could lead to the structure modification affected by PEF. But the effects of PEF on protein structure have received considerable attention in recent years (Castro, 1994; Fernández-Diaz et al., 2000; Barsotti et al., 2002; Giner et al., 2002; Perez & Pilosof, 2004; Li et al., 2005). Because the potential implementation of PEF in food preservation would be mainly related to liquid foods, it is necessary to study the possible changes in food proteins of liquid foods such as milk, soy milk or liquid egg. Available data about the effects of PEF on food proteins is mainly focused on egg white protein and β-lactoglobulin (Perez & Pilosof, 2004; Mañas & Vercet, 2006).

PEF effects on enzymes or food proteins appear to depend on the characteristics of the PEF system used, and on the electric process parameters (electric field intensity, number of pulses and pulse width and treatment time) (Barsotti & Chefel, 1999). Because of the different PEF conditions, the results for the PEF effects on the enzyme and proteins are different.

Fernández-Dıaz et al. (2000) showed that PEF treatments (electric field intensity of 27- 33 kV cm^{-1}, 50-400 pulses, pulse width of 0.3 μs) induced at least partial unfolding of ovalbumin (2% w/v), thus exposing all four S-H groups to the surface. Alternatively, PEF could have enhanced ionization of S-H groups into S-S, the ionized form being more reactive. The extent of S-H reactivity increased with dissipated energy. However, the results showed that high voltage exponential decay pulses did not induce permanent modifications of ovalbumin but only induced the reversible modifications of ovalbumin.

Jeantet et al. (1999) investigated the PEF treatment (exponential decay pulses and up to 35 kV cm^{-1}) effect on dia-ultrafiltered egg white. Prior dia-ultrafiltration was necessary to decrease the electric conductivity of egg white and obtain a high electric field across the electrodes. Surface hydrophobicity, as measured by ANS (1-anilino-8-naphtalene sulfonic acid) binding on aromatic amino acid residues, did not increase after PEF treatment, in contrast to the effects of heating (55^{o}C, 15 min). It was thus suggested that PEF treatment did not modify the structure of egg white proteins, although *Salmonella enteridis* was significantly inactivated.

Barsotti et al. (2002) confirmed that the exponential decay pulses delivered by the PEF system (30 kV cm^{-1} and pulse width of 1.3 μs) did not cause significant protein unfolding or aggregation when tested on solutions of ovalbumin or β-lactoglobulin (8-12%, pH 7), two proteins known to be sensitive to heat or high pressure processing. In contrast, Perez & Pilosof (2004) found that PEF induced structural changes in proteins and modified their functional performance. Two protein systems, β-lactoglobulin (10%, pH=7) and fresh egg white, were subjected to between 1 and 10 pulses of 12.5 kV/cm. Both β-lactoglobulin and egg white were increasingly denatured by enhancing the number of pulses.

2.1.5 Application of PEF in the food industry

There is a growing interest in the application of pulsed electric field (PEF) in food processing (Barbosa-Cánovas et al., 1999). Generally the application of PEF in food processing includes liquid foods and solid foods. An application of PEF for food preservation provides the tremendous potential to preserve high quality products at lower temperatures and short residence times to retain the fresh-like character and nutritional values of the products (Toepfl et al., 2005).

2.1.5.1 PEF applied to processing of liquid foods

The modern food industry is aware of the promising results of PEF treatments of apple juices, grape juices, orange juices, as well as milk and liquid egg products (Barbosa-Cánovas et al., 1999). Some of these PEF-treated foods showed an extension in shelf life of more than 8 weeks without refrigeration, which is one of the most attractive attributes of the technology. PEF treatments have been mainly applied to preserve the quality of foods such as milk, orange juice, and apple juice. Most of the results on liquid foods have shown that PEF treatments had few effects on the organoleptic properties of foods, compared to traditional thermal pasteurization and other pasteurization processes. Some specific products that have been studied include milk and milk products (Martin-

Belloso et al., 1997; Reina et al., 1998; Fleischman et al., 2004); apple juice (Qin et al., 1995b; Simpson et al., 1995; Vega-Mercado et al., 1997; Ortega-Rivas et al., 1998; Evrendilek et al., 2000; Zárate-Rodriguez et al., 2000); orange juice (Sitzmann, 1995; Zhang et al., 1997; Jia et al., 1999; Yeom et al., 2000a, b); liquid egg (Jeantet et al., 1999 & 2004; Fernández-Diaz et al., 2000; Hermawan et al., 2004; Perez & Pilosof, 2004).

2.1.5.2 Enhancement of the mass transfer in plant materials

Applying PEF on plant tissues as a pre-treatment can enhance subsequent processes such as juice extraction (Bazhal & Vorobiev, 2000; Bouzara & Vorobiev, 2000; Eshtiaghi & Knorr, 2002; Jemai & Voroviev, 2002) and dehydration processes (Rastogi et al., 1999; Ade-Omowaye et al., 2000; Taiwo et al., 2002).

Bazhal & Vorobiev (2000) studied the use of PEF on intensifying juice pressing from apple cassettes. PEF treatments consisted on applying 1 to 100 pulses and electric field intesnity of 0.2 to 1 kV, with the pulse width of 100 μs. The pulse wave form was square wave form. The author reported a significant incraese in apple juie yield with incraesing the number of pulses and/ or electric field intensity.

Bouzara & Vorobiev (2000) reported extraction of beet juice by pressing and PEF with potentials between 0 to 1.5 kV, 1 to 100 pulses and pulse width of 10 μs to 1 ms. The authors reported significant increase in the beet juice with a good quality for final juice product.

Ade-Omowaye et al. (2000) examined the impact of PEF on the cell permeabilization of coconut. The authors used PEF conditions which consisted of the electric field intensity of 0.1 to 2.5 kV cm^{-1}, pulse width of 575 μs and 0 to 200 pulses. Optimum PEF parameters (20 pulses, pulse width of 575 μs and electric field intensity of 2.5 kV cm^{-1}) were compared with other methods of cell disintegration. Coconut samples pre-treated with optimum PEF parameters resulted in a reduction of approximately 22% of drying time compared to untreated samples.

Eshtiaghi & Knorr (2002) studied the application of PEF-treatments (20 pulses and electric field intensity of 2.4 kV cm^{-1}) and different pressing methods. The results confirmed that PEF pre-treatment reduced the energy and time required for sugar beet extraction compared to the conventional thermal processes.

Jemai & Voroviev (2002) investigated the effect of PEF on diffusion coefficient of soluble substances from apple slices. The PEF treatment consisted of 1000 pulses, pulse width of 100 μs and electric field intensity of 0.5 kV cm^{-1}. It was found that PEF increased the diffusion of soluble substances from apple slices. Electric field intensity of 0.1 to 0.15 kV cm^{-1} started showing a significant effect of diffusion coefficients.

2.1.6 Advantages of PEF processing in the food industry

PEF processing is a nonthermal processing method used to increase shelf life and maintain food safety by inactivating spoilage and pathogenic microorganisms. This method is advantageous because the change in product color, flavor, and nutritive value is minimized during PEF processing (Jia et al., 1999; Ruhlman et al., 2001).

Use of PEF for inactivation of microorganisms is one of the more promising nonthermal processing methods for the replacement of traditional thermal pasteurization (Barbosa-Cánovas et al., 1998). The PEF process is a safe because no dangerous chemical reactions have been detected; moreover, it is reliable because the same results can be obtained repeatedly (Vega-Mercado et al., 1997). PEF treatment is associated with minimum energy utilization and high energy efficiency (Qin et al., 1995a). PEF treatment can increase the juice yields by the disruption of plant cell membranes and tissues (Angersbach et al., 1997).

2.2 FOOD PROTEINS AND THEIR STRUCTURAL AND FUNCTIONAL PROPERTIES

2.2.1 Whey proteins

Whey proteins mainly contain β-lactoglobulin (β-LG) (~50%), α-lactalbumin (α-LA) (~21%), immunoglobulins (10%), bovine serum albumin (BSA) (5%) and residual amounts of proteose-peptones (Kinsella, 1984). Whey proteins are normally stable to acid but sensitive to heat, especially β-LG. β-LG and α-LA are present in the highest concentrations and are primarily responsible for the physicochemical properties of whey proteins. These whey proteins have excellent functional properties, such as viscosity, gelation, foaming, solubility and emulsification (Kilara & Vaghela, 2004).

Whey proteins are an excellent source of all the essential amino acids and are easily digested. The major whey proteins in cow's milk are β-LG, α-LA, BSA and immunoglobulins (Havea et al., 1998; Herceg et al., 2002). Because of their nutritive value and functional properties, whey proteins can be used as main components in infant formula, weight-gain and weight-reduction diet foods, protein-fortified fruit juices and other healthy foods and drinks. Whey proteins have globular structures which are stabilized by intramolecular disulfide bonds between cysteine residues (Dickinson, 2001). Whey proteins are globular proteins which unfold and aggregate when they are heated higher than their denaturation temperature (de Wit, 1990; Sliwinski et al., 2003).

2.2.2 Soy proteins

Soy proteins are generally regarded as the storage proteins held in protein bodies which contain about 60-70% of the total soybean proteins. Approximately 90% of proteins in soybeans exist as storage proteins, which mostly consist of β-conglycinin and glycinin. Major soy proteins, namely, β-conglycinin and glycinin, possess a variety of functional properties for food applications (Fukushima, 2004). The increasing utilization of soy proteins is due to several factors including abundance, low cost, nutritional quality

and good functional properties including swelling, viscosity, gelation, emulsification and foaming and water retention (Zayas, 1997).

Soy protein isolate (SPI) is a highly refined or purified form of soy protein with a minimum protein content of 90% on a moisture-free basis. It is made from defatted soy flour which has most of the non-protein components, fats and carbohydrates removed. SPI is mainly used to improve the texture of meat products, but is also used to increase protein content, enhance flavor, and as an emulsifier.

2.2.3 Protein structure

2.2.3.1 Primary structure

Protein primary structure is defined as the sequence of 19 different α-amino acids and one imino acid residue in the peptide chain. Primary structure of a protein denotes the linear sequence in which the constituent amino acids are linked via peptide bonds. The chain length and the amino acid sequence of the polypeptide determine its ultimate three-dimensional structure in solution (Damodaran, 1997). Because peptide bonds constitute one third of all covalent bonds of the polypeptide backbone, the restriction on the rotational freedom drastically reduces the flexibility of the polypeptide chain. Most proteins assume a highly compact ordered structure because of steric factors and other noncovalent interactions among amino acid residues.

2.2.3.2 Secondary structure

Secondary structure relates to periodic structures in polypeptides and proteins. The most common secondary structures in proteins are the α-helix and the β-sheet. The α-helical structures in proteins are predominantly amphiphilic; one half of the helical surface is hydrophilic and the other half is hydrophobic (Damodaran, 1997). The β-sheet structure is an extended structure in which the C=O and N-H groups are oriented perpendicular to the direction of the backbone. In this configuration, hydrogen bonding

can occur only between sheets. Hydrogen bonds can lead to the conformation of some special types of secondary structure (Walstra et al., 2006).

2.2.3.3 Tertiary structure

The tertiary structure refers to the spatial arrangement of the entire polypeptide chain with secondary structure segments into a compact three-dimensional folded form. The folding of a protein into a compact tertiary structure is accompanied by a reduction in the interfacial area between nonpolar groups of the protein and the surrounding solvent water. The distribution of hydrophilic and hydrophobic residues and their relative fraction in the amino acid sequence influences several physicochemical properties, such as shape and solubility of the protein. Proteins containing a large number of hydrophobic residues tend to assume a globular shape so that more hydrophobic groups can be buried in the protein interior.

Further folding of protein secondary structure leads to the tertiary structure. Hydrophobic interactions are essential and a few internal salt bridges maybe formed. The tertiary structure of protein leads to globular proteins. Globular proteins can be formed if the primary structure contains a sufficient proportion of amino acid residues with a highly hydrophobic side group. Ideally, all the hydrophobic side groups are in the core of the folded molecule, all strongly hydrophilic ones at the outside and they are in contact with water (Walstra et al., 2006).

2.2.3.4 Quaternary structure

The quaternary structure refers to spatial arrangement of a protein. Each polypeptide chain is known as a subunit, and the quaternary complex is referred to oligomeric structure. The tertiary structure of proteins contains nonpolar patches on the surface, and hydrophobic interaction of these patches with adjacent protein molecules in aqueous leads to formation of oligomeric structures. Formation of quaternary structure is primarily driven by the thermodynamic requirement to bury exposed hydrophobic

patches. Other noncovalent interactions, such as electrostatic interactions and hydrogen bonding, at the interface of the subunits may also contribute to the stability of the quaternary structure.

2.2.4 Protein denaturation and protein modification methods

Denaturation of food proteins has been defined as a process in which the partial arrangement of polypeptide chains within the molecule is changed from that typical of the native protein to a more disordered arrangement (Kauzmann, 1959). Cheftel et al. (1985) defined protein denaturation more specifically as any modification in conformation (secondary, tertiary, or quaternary) not accompanied by the rupture of peptide bonds involved in primary structure.

Although each protein possesses a unique, well-defined structure in the native state, after denaturation, the same protein may have several nonspecific structures according to the type and extent of the denaturing treatment. These denatured structures are characterized by a degree of random configuration higher than that of the native molecule (Joly, 1965). Various levels of denaturation can be distinguished according to whether the secondary, tertiary, or quaternary structure of the protein is involved in the process.

Because elucidation of the structure-function relationship of proteins is the desired goal of many protein chemists and food scientists, the techniques of protein chemistry used for modifying proteins have centered on the parameters of hydrophobicity, steric forces, and electronegativity in evaluating the functional properties of proteins. Molecular properties of proteins such as surface hydrophobicity, net change, thermal properties and rheological properties are important for the functional properties of solubility, emulsification, foam formation and gelation, while steric factors of molecular size and flexibility are important for viscosity, coagulation and fat binding.

In discussing the effect of protein modification treatments on functional properties, emphasis is placed on protein structure and the role of molecular properties

such as hydrophobicity, molecular size and flexibility. There are some methods to effect protein structural modification and thus to improve the functional properties of proteins.

2.2.4.1 Physical modification

Physical modification of food proteins basically involves manipulation of conditions to achieve either a purer fraction of the desired component from a mixture, or to improve the performance of specific components in product applications. The influence of solvent conditions, such as pH, temperature and ionic strength on the surface active properties of several proteins has been actively studied in order to ascertain the relative importance of specific molecular attributes (Morr, 1975; Mita et al., 1978). Hydrophobic interactions during foam formation could decrease the foam stability of β-lactoglobulin with a decrease in temperature (Phillips & Kinsella, 1990).

2.2.4.2 Chemical modification

Several chemical modification reactions have been investigated to improve the functional properties of proteins such as solubility, viscosity, heat stability, surface active properties and gelation (Kinsella & Whitehead, 1987). The primary effect of chemical modification procedures is generally to increase protein solubility and functional properties such as foaming and emulsification in particular. Kim & Kinsella (1987) used chemical modification to decrease the rate of adsorption and improve the emulsifying capacity and activity and the film foaming properties of proteins by using succinylation and glycosylation. In addition to improving the functional properties of food proteins, chemical modification also enhanced the nutritional improvement of many proteins. Free amino acids, tryptophan and threonine have been incorporated into food proteins via phosphorylation and enzyme catalyzed covalent attachment reactions (Chobert et al., 1987).

2.2.4.3 Enzymatic modification

Enzymatic modification of proteins has a long tradition of use in improving the functional behavior of milk proteins for cheese making. Enzymatic modification can generally be performed under milder experimental conditions. Use of enzymes holds the advantage that the toxicity is reduced and greater stereo chemical specificity may be achieved (Arai & Watanabre, 1988). Improvement of protein solubility can be achieved through limited digestion of food proteins such as whey and soy proteins by adding food-grade proteases (Adler-Nissan, 1986). Limited treatment of whey protein concentrate solutions with proteases such as trypsin and papain has resulted in improved foaming properties of these proteins (Horiuchi et al., 1978).

2.2.4.4 Protein modification by high pressure treatments

The effects of high pressure on the structure and function properties of proteins have received attention because of their importance in the understanding of protein thermodynamics (Tanaka et al., 1996). Many studies have been done to understand the effect of high pressure on functional properties of single whey proteins, soy protein and caseins, such as gel formation (Famelart et al., 1998), emulsifying capacity (Galazka et al, 1996), foamability (İbanoglu & Karatas, 2001) and emulsifying properties and rheological properties of soy proteins (Floury et al., 2002). Some studies also have been carried out regarding the effect of high pressures on protein modification and functional properties of proteins, such as α-lactalbumin and β-lactoglobulin (Tanaka et al., 1996), whey protein concentrate (Liu et al, 2005), whey protein isolate (Bouaouina et al., 2006), soy proteins (Molina et al, 2001; Zhang et al., 2005) and egg white (Ahmed et al., 2003; der Plancken et al., 2007).

2.2.5 The relationship between protein structure and protein functionality

Functional properties of a protein in food are related to its physical, chemical and structural properties, which include size, shape, amino acid composition and sequence.

Most functional properties affect textural qualities of a food and play an important role in determining the physical behavior of a food during preparation, processing and storage. The three-dimensional structure of a protein defines how it functions in foods. Processing treatments such as heat, pH, salt or shear and subsequent storage conditions can cause protein denaturation and even unfolding of a food protein. Subtle changes in protein structure may affect its functionality in foods and result in changes in nutritional value, and textural and sensory properties. An understanding of the relationship between structure and functionality in proteins might lead to improvement of food products and the discovery of novel ingredients.

The physicochemical properties that affect functional properties of proteins are related to the size, charge distribution, hydrophobicity, hydrophilicity, molecular flexibility, and steric properties of proteins. Among these, molecular flexibility and hydrophobicity are considered to be the most important factors influencing several functional properties. Physical, chemical, and enzymatic modification methods can be employed to change these molecular properties. In order to apply such methods to improve functional properties of proteins, one needs to know how hydrophobic property and how flexible certain regions of a protein should be in order for it to exhibit improved functionality.

Hydrogen bonding plays a major role in increased viscosity, which precedes the onset of coagulation. This type of cross-link allows the open orientation necessary for water immobilization and may be the most important type of cross-linking in reversible gels (Schmidt, 1981). Protein solvation increases with increased salt addition due to decreased protein-protein attraction and increased protein-solvent attraction. At higher ionic strength, there is increased protein-protein attraction as the ions compete with the protein for solvent, which results in the formation of larger aggregates (Hermansson, 1979; Schmidt, 1981). Hydrophobic interactions are also involved in protein aggregation and gelation. Nonspecific hydrophobic interactions have been implicated in the dissociate-associative reactions that initiate aggregation and gelation and also in improved strength and stability of protein gels on cooling (Schmidt, 1981).

The relationship between protein structure and functionality continues to be an area of significant interest to food scientists and protein chemists. An understanding of protein denaturation and affected functional properties of proteins is imperative to the achievement of specific textural attributes in foods. Thermal treatments have been the most common processing techniques used in denaturing food proteins. Most of our understanding of the functional behavior of food proteins has been based on their thermal properties. Most recently, other processes, such as high pressure treatment and PEF treatments, have been used to denature proteins and to induce specific functional properties such as coagulation and gel formation. This is a growing area, which may hopefully should be understood the mechanisms of protein denaturation and functionality.

2.2.6 The main functional properties of food proteins

Functional properties of proteins are those physicochemical properties of proteins which affect their behavior in food systems during preparation, processing, storage, and consumption and contribute to the quality and sensory attributes of food systems (Kinsella, 1976). Functional properties are affected by hydrophobicity of proteins, molecular weight, protein structure and charge distribution on the protein molecules. The content of apolar amino acids (2.5-3.0% in most proteins) can affect protein structure, hydration, solubility and gelation properties. Charged amino acids on the protein molecule can enhance electrostatic interactions which stabilize the globular proteins and influence water binding. The compactness of the protein molecule structure and extent of bonding and interactions significantly affect the functional properties of proteins. The effect of protein structure on its functionality is demonstrated by globular proteins that have more charged amino acids toward the surface which accelerates solubility, swelling and hydration (Zayas, 1997). The common functional properties of proteins that are considered to contribute most to the characteristics of processed food include solubility, water holding capacity, foaming, emulsification and gelling ability.

Functional properties of a protein are affected by the composition and structure of the protein and by its interaction with environment factors. The following factors influence functional properties of proteins: pH of medium, treatment temperature, ionic strength, moisture content, oxidation/reduction potential, shear stress and shear rate (McGowen & Mellors, 1979), high pressure (Messens et al., 1997; López-Fandiño, 2006) and pulsed electric field (Perez & Pilosof, 2004). Knowledge of the relationship among protein structure, its physicochemical properties and functional properties under various environmental conditions is critical for its proper food usage and can be used to control functional properties of food proteins (Zayas, 1997).

2.2.6.1 Protein solubility

Protein solubility is required because of its influence on other functional properties. Protein solubility and water retention are critical factors in protein functionality because they affect texture, color, and sensory properties of food products. Protein solubility contributes to other functional properties such as emulsification, gelation and foaming of proteins and may be a prerequisite for efficient use of various proteins in food processing as functional food additives. Protein solubility depends on temperature, pH, protein concentration and solvent properties in the system and surface hydrophobicity (Zayas, 1997). The protein solubility is affected by the amino acid composition and structure and hydrophobic and hydrophilic properties. Protein-protein interaction in an aqueous medium is accelerated by hydrophobic interactions between the nonpolar groups in the protein. The average hydrophobicity of amino acid residues and charge frequency are the two most important factors that determine the solubility of proteins: the lower the average hydrophobicity and the greater the charge frequency, the greater the solubility (Damodaran, 1997).

2.2.6.2 Water holding capacity (WHC)

Water holding capacity (WHC) is generally referred to as the ability of food to retain its natural water as well as added water during processing and is a functional

property of protein-based foods. The WHC of proteins affects food characteristics such as texture, juiciness and taste (Xiong, 1997) and it is the ability to retain water against gravity physically and physicochemically. Protein-water interactions determine functional properties of proteins in foods such as: water binding and retention, swelling, solubility, emulsifying properties, viscosity, gelation and syneresis. The WHC plays a major role in the formation of food texture, especially in comminuted meat products and baked doughs (Zayas, 1997). The WHC of food proteins is influenced by several intrinsic factors such as the type of protein, protein structure, and amino acid composition and by several extrinsic factors such as pH, ionic strength and temperature.

2.2.6.3 Emulsification

An emulsion is a mixture of two or more immiscible liquids (usually oil and water), with one of the liquids dispersed as small spherical droplets in the other liquid. Emulsions can be conveniently classified according to the distribution of the oil and aqueous phases. A system, which consists of oil droplets dispersed in an aqueous phase, is called an oil-in-water or O/W emulsion such as milk, cream, mayonnaise, soups and sauces. A system which consists of water droplets dispersed in an oil phase is called water-in-oil or W/O emulsion such as margarine and butter (McClements, 1999). Food emulsions are quite complex because of the presence of other compounds contained in different phases (Phillips et al., 1994).

Emulsification is the most important process in the manufacturing of many formulated foods. The understanding of the emulsifying properties of food proteins is enhanced by establishing a correlation between surface hydrophobicity and interfacial tension. Proteins with a large number of apolar amino acids, i.e., high hydrophobicity are surface active. Emulsifying properties of proteins depend on their solubility, hydrophilic/hydrophobic balance, molecular flexibility and the nature and proportion of lipid and other non-protein components in the system (Tornberg et al., 1990). Emulsifying properties of proteins closely depend on their surface hydrophobicity (Kato & Nakai, 1983), and surface hydrophobicity has been proposed as a useful parameter in

predicting emulsifying properties of food proteins. Many hydrophobic residues of proteins are buried in the interior of the native protein and which may have little relevance as a predictor of the capacity of a protein to interact with water and oil in an emulsion system (Phillips et al., 1994). Other factors which affect emulsifying properties include temperature, pH and the type of protein. The pH of emulsion formation is an important criterion in determining the amount of protein that is adsorbed at the interface and affects the stability of the emulsion that is formed (Pearce & Kinsella, 1978).

Whey proteins have a great surface hydrophobicity and they improve their emulsifying capacity by moderate heating and partial unfolding. Proteins must possess a well-balanced distribution of hydrophilic and hydrophobic domains in the molecules. An effective method of surface hydrophobicity determination is the use of fluorescence probes which bond to the hydrophobic regions on the surface of the protein (Kato & Nakai, 1983). Since the surface hydrophobicity of protein increases with denaturation at the interface, the emulsifying properties may be improved. Solubility and functionality might also be improved by protein modification. Solubility and surface activity of modified proteins are improved by partial hydrolysis during enzymic modification or by heat treatments (Li-Chan et al., 1984).

2.3 FLUORESCENCE SPECTROSCOPY

2.3.1 Introduction

Fluorescence spectroscopy provides detailed information about the composition and molecular structure of proteins in food systems. This technique is based on molecular interactions with electromagnetic radiation that result in transitions between the vibrational energy levels of the ground electronic energy state of the molecule, corresponding to the excitation of various stretching and bending vibrations.

Fluorescence spectroscopy has the main advantage over other optical techniques of being extremely sensitive. Many applications of fluorescence spectroscopy rely on this fact to label small amounts of sample for detection, often allowing solute in the nanomolar range to be quantified. The fluorescence of many fluorophores is extremely

sensitive to their surrounding environments, e.g., tryptophan in proteins. This enables fluorescence to be used as a sensitive structural probe in many biological systems using only small amounts of sample compared to other spectroscopic techniques.

2.3.2 Fundamental principle of fluorescence spectroscopy

Fluorescence, particularly intrinsic protein fluorescence, is extremely sensitive to temperature. Fluorescence experiments therefore always should be carried out with the cell holder connected to a thermostatically controlled circulator, which can accurately maintain the desired temperature. Probably the most important consideration in the preparation of a sample for fluorescence is its concentration. The sample should have an absorbance of ~0.1 at the excitation wavelength. This is because of the inner filter effect. This effect increases greatly with increasing absorbance of the sample, and can decrease the fluorescence intensity by either lowering the effective intensity of the exciting light or absorbing some of the fluorescence.

Fluorescence data are generally presented as emission spectra. A fluorescence emission spectrum is a plot of the fluorescence intensity versus the emission wavelength (nanometers, nm). Emission spectra vary widely and are dependent on the chemical structure of the fluorophore and the solvent in which it is dissolved. Emission spectra are typically independent of the excitation wavelength (Lakowicz, 2006).

The measurements of protein fluorescence can be used to study any phenomenon that causes changes to the tryptophan environment. This includes protein folding or unfolding and association reactions. Measurements of emission spectra give information on the accessibility of the fluorophore to solvent characteristic of its environment. Changes in the environment will change both the emission maxima and the fluorescence intensity as the tryptophan is exposed/protected by solvent and quenched/unquenched by nearby groups. The fluorescence intensity can be followed by measurement of the fluorescence at a single wavelength. The change in emission maximum is followed by measurement of the emission spectrum.

2.3.3 Fluorescence spectroscopy of food proteins

Fluorescence spectroscopy has found extensive application in the study of proteins and is particularly useful technique for the elucidation of protein tertiary structure. Fluorescence probes represent the most important area of fluorescence spectroscopy. The wavelength required for the instruments is determined by the spectral properties of the fluorophores (Lakowicz, 2006).

The most dramatic aspect of fluorescence is its occurrence at wavelengths longer than those at which absorption occurs. The Stoke shift describes the observation that fluorescence photons are longer in wavelength than the excitation radiation (So & Dong, 2002). These Stokes shifts, which are most dramatic for polar fluorophores in polar solvents, are due to interactions between the fluorophore and its immediate environment. The indole group of tryptophan residues in proteins is one such solvent-sensitive fluorophore, and the emission spectra of indole can reveal the location of tryptophan residues in proteins. The emission from an exposed surface residue will occur at longer wavelengths than that from a tryptophan residue in the protein interior.

2.3.3.1 Intrinsic fluorescence of proteins

Proteins contain three aromatic amino acid residues (tryptophan, tyrosine, and phenylalanine) which may contribute to their intrinsic fluorescence. Changes in intrinsic fluorescence can be used to monitor structural changes in a protein. The fluorescence of a folded protein is a mixture of the fluorescence from individual aromatic residues. Protein fluorescence is generally excited at 280 nm or at longer wavelengths. Most of the emissions are due to excitation of tryptophan residues, with a few emissions due to tyrosine and phenylalanine (Kulmyrzaey & Defour, 2002; Garimella-Purna et al., 2005).

The emission of tryptophan is highly sensitive to its local environment, and is often used as a reported group for protein structural changes. Spectral shifts of protein emission have been observed as a result of several phenomena, including protein-protein association and protein unfolding. The emission maxima of proteins reflect the average exposure of their tryptophan residues to the aqueous phase.

2.3.3.2 Extrinsic fluorescence of proteins

Extrinsic fluorescence is generally the addition of fluorescent dyes to a system in order to study them. The use of such compounds generally reflects the limitations of the use of intrinsic fluorophores to study biological systems. The use of fluorescent compounds to study structure and dynamic systems is also extremely varied.

A valuable property of many fluorophores is their sensitivity to the surrounding environment. The emission spectra and fluorescence intensities of extrinsic probes are often used to determine probes' location on a macromolecule. For example, one of the widely used probes for such studies is l-anilino-8-napthalenesulfonic acid (ANS), which displays the favorable property of being very weakly fluorescent in water. Weak fluorescence in water and strong fluorescence when bound to a protein molecule are convenient properties shared by other widely used probes.

ANS is one of the most widely used probes. This probe has a 100-fold fluorescence enhancement and a significant spectral blue shift as well as a longer emission decay upon transfer from water to a non-polar environment when it occurs in binding to a protein's hydrophobic core. Therefore, the binding characteristics of ANS to protein and its spectral properties deduced from the ANS fluorescence change can be used to explore protein hydrophobicity, structural modifications and unfolding. ANS has a higher binding affinity to the pressure-induced molten globule state of protein (Clery et al., 1995; Masson & Clery, 1996). The same phenomenon was also observed for pressure-modified trypsin (Ruan et al., 1997; Ruan et al., 1999).

2.3.3.3 Application of intrinsic fluorescence in the study of protein structure

Most proteins are endowed with an intrinsic fluorescence because they contain aromatic amino acids, particularly tryptophan. Tryptophan has the highest fluorescence quantum yield overshadowing markedly the emissions of the other two intrinsic fluorescences. Tryptophan emission maxima in proteins can vary from 332 nm to 342 nm depending on the protein. Free tryptophan has a characteristic fluorescence emission at 350-360 nm.

Karamonová et al. (2003) studied the relationship between thermally-induced changes in tertiary structure of whey proteins which were heated and then cooled to room temperature using the intrinsic fluorescence. On heating and then cooling the emission wavelength of the maximum tryptophan fluorescence intensity of whey proteins showed a red shift, moving to longer wavelengths. For α-lactalbumin (α-LA), the excitation wavelength of 291 nm and the maximum tryptophan fluorescence intensity of emission spectra showed such a red shift after heating the protein to the temperature of 80^{o}C, reaching a plateau value of 352.5 nm after heating to 110^{o}C, compared with the maximum tryptophan fluorescence intensity of 333 nm for the unheated protein α-LA. The maximum tryptophan fluorescence intensity of emission spectra of β-LG variants A and B also showed a red shift from 340 to 346 or 347 nm after heating to the temperature of 90^{o}C, and tailing off at around 110^{o}C for variants A and B, respectively.

Palazolo et al. (2000) found an alteration in β-LG fluorescence emission on its modification with heat treatment. The heat treatment of β-LG resulted in a number of irreversible, linked, spectrally detectable changes to the protein structure. The structural modification resulted in the loss of the hydrophobicity of the tryptophan environment, and variations in thiol group availability (Creamer, 1995; Manderson et al., 1999). After the protein was treated, the emission wavelength of the maximum intrinsic fluorescent intensity was shifted from 332 nm to longer wavelengths. This red shift indicated that the major tryptophan fluorophore (Trp-19) had moved from an apolar environment to a more polar region. Moreover, the quantum yield of β-LG increases when the protein is denatured by heat treatment, presumably because the decrease in quenching of Trp-61, becomes more distant from the Cys-66-Cys-160 disulfide bond. Consequently, in the unfolded state, the solvent exposure of both Trp-19 and Trp-61 could be approximately the same. Therefore, both tryptophan residues could be similarly quenched by acrylamide with a higher efficiency than in the native state of the protein. In conclusion, the more intense the heat treatment and the higher is the degree of denaturation of whey protein.

Liu et al (2005) conducted detailed studies about the change of the intrinsic fluorescence intensity of whey protein concentrate (WPC) under high pressure

treatments. The fluorescence intensity increased and a red shift was observed after high pressure (HP) treatments, which indicated changes in the polarity of whey protein tryptophan residues' microenvironment of whey proteins from a less polar to a more polar environment. High pressure treatment with no come-up time resulted in a 2 nm red shift of the maximum emission wavelength from 335 nm to 337 nm. After HP treatment of 2.5 min, there was a 1.1-fold increase in fluorescence intensity and the emission wavelength of the maximum fluorescence intensity shifted from 335 to 338 nm. HP treatment of 30 min resulted in a further shift of emission wavelength from 335 to 339 nm and a final 1.2-fold increase in intrinsic fluorescence.

Stapelfeldt & Skibsted (1999) found that the pressure unfolding of β-LG increased its intrinsic fluorescence intensity. The emission wavelength of the maximum fluorescence intensity was red shifted under pressure. Although the spectral shift paralleled the change in the fluorescence intensity, there was some indication in the spectra of a pre-denaturation stage during which the fluorescence intensity increased without a significant spectral shift (Stapelfeldt et al. 1996). Moreover, the spectral shift continued to change at pressures greater than 200 MPa, while the fluorescence intensity remained constant.

2.3.3.4 Application of extrinsic fluorescence in the study of protein structure

The structural modifications that lead to protein denaturation include the exposure of hydrophobic sites previously buried inside the native structure of protein molecules (Moro et al, 2001). In general, protein denaturation results in an increase of hydrophobicity, due to exposure of hydrophobic groups that are folded inside the intact native protein molecule (Mine, 1997). Thus, measurements of the surface hydrophobicity are useful in predicting the extent of milk protein denaturation and the functionality of milk proteins in milk. Fluorescence titration employing hydrophobicity probes such as 1-anilino-8-naphthalenesulfonate (ANS) has successfully been used to determine the surface hydrophobicity of whey proteins or other proteins (Moro et al., 2001).

For the majority of proteins, Surface hydrophobicities increase when the protein molecules unfold due to heat treatment or high pressure treatment (Moro et al., 2001; Zhang et al., 2005). Protein denaturation can occur in various ways, all in relation to structural modifications. Among these, changes in the surface hydrophobicity of proteins, taking place in the early steps of the denaturation process, are often measured by following the binding of ANS to accessible aromatic hydrophobic sites (Hayakawa & Nakai, 1985; Bonomi & Iametti, 1991).

2.4 THERMAL PROPERTIES OF FOOD PROTEINS

Knowledge of thermal properties (e.g. specific heat Cp, enthalpy ΔH, thermal conductivity and thermal diffusivity) of food proteins may help to predict heat transfer rates in food proteins. Specific heat indicates how much heat is required to change the temperature of a food protein. It depends strongly on the temperature and composition (such as moisture content, fat content and the nature of the solid component, e.g. protein and carbohydrates and other components) of the food product (Ngadi et al., 2000). Enthalpy is the heat content or energy level of a material. Generally it is defined as the absolute value of enthalpy, a zero value is usually arbitrarily defined at -40^{o}C, 0^{o}C, or some other convenient temperature. Enthalpy has been used to predict quantifying energy in foods and it can be used to calculate the amount of heat energy required to heat a material from one temperature to another temperature (Sweat, 1986).

Differential scanning calorimetry (DSC) is a powerful technique to study the thermodynamics of protein stability that could provide a basic understanding of protein denaturation (Kurganov et al., 1997; Thorarinsdottir et al., 2002). DSC has been established as a technique for studying thermal denaturation and conformational transition of proteins and it has been used in the study of various food systems (Arntfield et al., 1990; Chan et al., 1992; Lu et al., 2002; Raemy, 2003; Tang, 2007). DSC can detect the thermal denaturation of a protein in a complex protein system as an endothermic peak in its thermogram (Katsuji et al., 1988; Boye, 1995). DSC has emerged as the technique of choice for the study of thermal transitions of food. In this technique,

the substance of interest and an inert reference are heated at a programmed rate. Any thermally induced changes occurring in the sample are then recorded as a differential heat flow displayed normally as a peak on a thermogram. DSC can be used to monitor changes in denaturation temperature (T_d) and enthalpy of the transition (ΔH) of macromolecules such as proteins by detecting the heat changes associated with such processes. Proteins may undergo denaturation under various buffer conditions, attributed to the disruption of chemical forces that maintain the structural integrity of protein molecules, and these can be exhibited in the DSC thermograms (Meng & Ma, 2001). Boye et al. (1997) studied the thermal and structural stability of α-lactalbumin in response to sugars using DSC. Protein-metals and protein-salts interactions have also been studied by DSC (Relkin et al., 1993).

2.5 RHEOLOGICAL PROPERTIES OF FOOD PROTEINS

Food rheology can be used to characterize not only flow behavior of biological and food materials, but also structural characteristics. Flow properties, such as viscosity, yield stress, thickness and consistency index contribute substantially to facilitate transport and commercial processing, as well as to promote consumer acceptance. Insight on structural arrangement helps to predict behavior or stability of a food material with storage, change in humidity and temperature and handling (Motyka, 1996). Measuring the rheological properties and identifying the rheological behavior of food materials is necessary when employing any of the available commercial instruments that allow objective characterization. The understanding of rheological behaviors of foods has been recognized to be of importance in the following steps during manufacturing: quality control of ingredients and finished food products; design and evaluation of process and unit operations equipment; characterization and development of food products for consumer acceptability; and elucidation of the structure and the relationship among structure and textural properties (Velez-ruiz, 1996).

Most of the rheological characterization of milk products and food protein solutions behaved as non-Newtonian fluids with linear regression analyses resulting in

values of slopes corresponding to the flow behavior index (n) and intercepts that allow the evaluation of the consistency index (K) showing high correlation coefficients (0.99). The rheological nature of milk at low concentration levels fits Newton's equation, whereas the non- Newtonian nature fits one of two equations (Power Law model or Herschel-Bulkley model) depending upon the yield stress which is detected (Velez-ruiz, 1996).

According to Rha (1978), liquid food products show diverse flow behaviors (dependence on shear rate) and thus it is necessary to critically analyze the nature of the response under a variety of flow conditions. Rheological modeling can be used to interpret rheological data in terms of mathematical expressions. Flow behavior is visualized as a plot of shear stress versus shear rate, and the resulting curve is mathematically modeled using various functional relationships. The simplest type of substance is the Newtonian fluid where shear stress is directly proportional to shear rate. (Steffe, 1996). A general relationship to describe the behavior of non-Newtonian fluids is the Herschel-Bulkley model:

The non-Newtonian fluids are generally described by the Hershel-Burkley model:

$$\tau = K(\gamma)^n + \tau_o \qquad (5)$$

where K is the consistency index (Pa.s^n), n is the flow behavior index, τ is the shear stress (Pa), γ is the shear rate (s^{-1}) and τ_o is the yield stress (Pa) (Steffe, 1996). This model is appropriate for many fluid foods. The consistency index (K) represents viscosity (μ) and plastic viscosity (μ_p) for Newtonian and Bingham Plastic fluids, respectively. The flow behavior index (n) equals to 1 for Newtonian and Bingham plastic fluids. The Power Law fluids have shear thinning behavior (pseudoplastic) when $0< n <1$ or shear thickening (dilatent) behavior when $1< n <\infty$. Hershel-Bulkley and Bingham plastic fluids are characterized by the presence of a yield stress (σ_o) that represents the minimum stress required to initiate the flow of the material (Dias-Morse, 2004).

Numerous factors influence the selection of the rheological model used to describe flow behavior of a particular fluid. Apparent viscosity decreases with increasing

shear rate in the shear-thinning and Bingham plastic substances. In Herschel-Bulkkey fluids, apparent viscosity will decrease with higher shear rates when $0< n <1$. Apparent viscosity is consistent with Newtonian materials and increases with increasing shear rate in shear-thickening fluids. A single point apparent viscosity value is sometimes used as a measure of mouth-feel of fluid foods. The human perception of thickness is correlated to the apparent viscosity at approximately shear rate of 60 s^{-1} (Steffe, 1996).

Proteins are very important in food systems and they contain the amino acids essential for human metabolism and contribute a wide range of functional properties that modulate the properties and attributes of foods. The functional properties of proteins depend on a specific linear sequence of amino acids and the corresponding three-dimensional structure. The detailed three-dimensional structure results from complex interactions between the specific components of the sequence and their solvents.

Heat treatment of proteins often results in increased water uptake and swelling as proteins unfolded. This results in an increase in hydrodynamic volume and increased resistance to flow (Kinsella, 1984). The increase in viscosity also reflects intermolecular interactions resulting from attractions between adjacent molecules with the formation of weak transient networks. Where there is no interaction between particles, flow properties depend only on the volume fraction or concentration of the suspended material and show Newtonian behavior. In concentrated solutions the hydrodynamic domains of the protein molecules come into contact, resulting in interactions between suspended particles. Concentrated protein solutions exhibit non-Newtonian behavior and show viscoelastic properties (Rha, 1978; Rha & Pradipasena, 1986).

The consumer acceptability of several liquid and semi-solid-type foods (e.g., gravies, soups, beverages) depends on the viscosity or consistency index of the products. The viscosity or consistency index of solutions is greatly influenced by solute type. Large molecular weight soluble polymers greatly increase viscosity even at low concentration. This depends on several molecular properties such as size, shape, flexibility, and hydration. Solutions of randomly coiled polymers display greater viscosity than do solutions of compact folded polymers of the same molecular weight. Some proteins, such

as gelatin, myosin, which have large axial ratios exhibit high viscosity even at low concentration.

The viscosity of protein solutions generally increases exponentially with protein concentration; this is attributable to increased interaction between the hydrated protein molecules. The ability of the protein to absorb water and swell affects its viscosity. Partial denaturation and/or heat-induced polymerization, which cause an increase in the hydrodynamic size of proteins, increase viscosity. The viscosity of some protein solutions, e.g., β-lactoglobulin, increases with time when the solution is sheared at a constant shear rate. This is attributable to shear-induced partial denaturation and sulfhydryl disulfide-induced polymerization of β-lactoglobulin (Phillips et al., 1994).

Solution conditions, such as pH, ionic strength, and temperature, affect viscosity of protein solutions. The viscosity of globular protein solutions generally decreases as the pH is decreased toward the protein's isoelectric pH. Partial thermal denaturation generally increases the viscosity of commercial whey protein concentrate (WPC). Increase of ionic strength generally decreases the viscosity of protein solutions by affecting their hydration capacity.

CONNECTING TEXT

The preceding review of literature demonstrated the need for the study of pulsed electric fields (PEF) effects on structural modification and surface hydrophobicity of food proteins. Through the use of fluorescence spectroscopy, the following study sought to determine the structural modifications and changes in surface hydrophobicity of whey protein at different protein concentrations, when exposed to different electric field intensities and different pulses.

Chapter 3 describes how the structural modification and surface hydrophobicity of whey protein isolate (WPI) exposed to a factorial combination of three levels of electric field intensity (12, 16 and 20 kV cm^{-1}) and three levels of number of pulses (10, 20 and 30), were evaluated using fluorescence spectroscopy. The current study confirmed that PEF treatments made the structural modification of whey protein and increased the surface hydrophobicity of whey protein.

3. PULSED ELECTRIC FIELD-INDUCED STRUCTURAL MODIFICATION OF WHEY PROTEIN

3.1 ABSTRACT

Effects of pulsed electric fields (PEF) on structural modification and surface hydrophobicity were assessed for whey protein isolate (WPI) of protein concentrations (3% and 5%) using fluorescence spectroscopy. The effects of a factorial combination of electric field intensities (12, 16 and 20 kV cm^{-1}) and number of pulses (10, 20 and 30) on intrinsic tryptophan fluorescence intensity, and extrinsic fluorescence intensity of 1-anilino-naphthalene-8-sulfonate (ANS) probe, served in qualifying protein structural modification and increases in surface hydrophobicity of WPI were evaluated. PEF treatments of WPI resulted in the increase of intrinsic tryptophan fluorescence intensity and 2-4 nm red shifts in the emission spectra, indicating changes from a less to a more polar microenvironment of tryptophan residues in whey proteins. With PEF treatments, extrinsic fluorescence intensity of WPI increased, but with 2-4 nm blue shifts, indicating partial denaturation of WPI fractions and exposure of more hydrophobic regions under these PEF treatments. Thus, under the conditions applied, PEF treatments of WPI yielded increases in surface hydrophobicity. The results of this study confirmed that PEF treatments increased intrinsic fluorescence intensity, extrinsic fluorescence intensity and surface hydrophobicity of whey protein.

Keywords: Pulsed electric fields (PEF), whey protein isolate (WPI), protein structure, intrinsic fluorescence intensity, surface hydrophobicity

3.2 INTRODUCTION

Whey proteins and their products, including whey protein isolate (WPI) have high nutritional value and functional properties. They are used in the food industry to improve

solubility, to facilitate whipping and improve the emulsifying, foaming and aeration properties of many formulated food products (Xiong et al., 1993). The impact of hydrophobic interactions of food proteins on protein functional properties has received major attention (Semisotnov et al., 1991). The functional properties of proteins are closely related to their surface hydrophobicity, emulsion capacity and emulsion stability of the proteins. Increased fat binding capacity was associated with an increase in hydrophobicity of the protein (Voutsinas & Nakai, 1983). Modifications of proteins that enhanced surface hydrophobicity showed promise for improving flavor properties of foods (Liu et al., 2005).

Pulsed electric field processing is a unique technique which offers many advantages over conventional thermal processing; including the ability to retain many of the food's quality attributes which may be lost in thermal processing (Quass, 1997). Several studies related to the effects of PEF on the inactivation of microorganisms and enzymes of liquid foods have been reported (Barbosa-Cánovas et al., 1999; Jeantet et al., 1999; Heinz et al., 1999; Wouters et al., 2001; Yeom & Zhang, 2001). Electric field intensity, pulse width and number of pulses are the main factors affecting both enzyme inactivation, and general modifications of protein structure, including unfolding of monomers, aggregation, formation of gel structures, and outright denaturation (Ho et al., 1997; Vega-Mercado et al., 1997; Barbosa-Cánovas et al., 1999; Barsotti et al., 2002; Perez & Pilosof, 2004; Zhong et al., 2005a; Loey et al., 2002). However, in comparison to the extensive research devoted to the inactivation of microorganisms and enzymes by PEF, only limited information is available concerning the effects of PEF on the structural modification of food proteins. Indeed, PEF could be used to modify the structure/function of proteins in order to achieve specific and/or desired functionality (Perez & Pilosof, 2004; Zhong et al., 2005b; Li et al., 2007). In a manner similar to the use of controlled heat treatments to improve aspects of protein functionality such as water absorption and mimic fat characteristics (Miller, 1994).

Fluorescence spectroscopy is a sensitive and versatile optical technique for studying the structure, dynamics, and interactions of proteins in solution (Eftink, 1994; Herbert et al., 1999). Fluorescence spectroscopy makes it possible to monitor changes in

tryptophan residue that result from changes in protein structure and interactions (Herbert et al., 1999). In fluorescence studies of proteins the fluorophores used can be either intrinsic (tryptophan, tyrosine or phenylalanine) or extrinsic probes [anionic aliphatic (cis-parinaric acid or CPA) or aromatic (1-anilinonaphthalene-8-sulfonic acid or ANS)] (Alizadeh-Pasdar & Li-Chan, 2000). Intrinsic fluorescence of most proteins is due to the presence of fluorescent aromatic amino acids, such as tryptophan, tyrosine or phenylalanine (Eftink, 1991; Eftink, 1994). Tryptophan residues are particularly valuable probes since the indole ring is very sensitive to its environment and there are often a limited number of tryptophan residues in a given protein. In general, protein structural modification results in an increase in hydrophobicity, due to the exposure of groups normally folded within the intact native protein molecule (Mine, 1997). Extrinsic fluorescence materials, including hydrophobicity probes such as ANS, have been successfully used to determine the surface hydrophobicity of whey proteins (Moro et al., 2001; Liu et al., 2005). With extrinsic fluorescence the probes undergo changes in one or more fluorescence properties as a result of noncovalent interaction with proteins (Semisotnov et al., 1991). Thus, measurements of extrinsic fluorescence intensity are useful in predicting the extent of protein structural modification.

A limited number of studies have reported the effect of PEF on the structure and functionality of single enzymes or proteins: β-lactoglobulan (Perez & Pilosof, 2004), ovalbumin (Fernández-Díaz et al., 2000), egg white (Jeantet et al., 1999), horseradish peroxidase (Zhong et al., 2005b) and pectinesterase (Zhang et al., 2006). The extent of investigations into the effects of PEF on structural modification of whey protein isolate (WPI) is very limited. Consequently, the objective of this study was to investigate the PEF treatments how to affect on structural modification of whey protein.

3.3 MATERIALS AND METHODS

3.3.1 Sample preparation

BiPRO whey protein isolate (WPI) powder (lot no. JE348-6-440) was provided by Davisco Foods International, Inc. (Le Sueur, MN). The product contained 93% protein

(β-lactoglobulin and α-lactalbumin), 0.2% fat, 2% ash, and 4.8% moisture, as measured by the supplier's standard proximate analysis procedures. The analysis was not repeated in our laboratory. The pH of a 10% (w/v) WPI solution at 20^{o}C was 7.1. The fluorescence spectroscopy probe, 1-anilino-8-naphthalenesulfonate (ANS) was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). All other chemicals (e.g. HCl and buffer solutions) were of analytical grade, purchased from Fisher Chemicals (Fairlawn, NJ), unless otherwise specified.

3.3.2 Preparation of samples

Whey protein isolate solutions (3%, 5%, w/v) were prepared using distilled water, and adjusted to pH 7.0 ± 0.1 using 1M HCl. These concentrations were used to facilitate PEF treatments, since low concentration protein solutions exhibit a high electrical resistance, such that greater pulse widths are necessary (Barsotti et al, 2002).

3.3.3 Experimental design

Two levels of WPI of protein content (3% and 5%), three levels of electric field intensity (12, 16 and 20 kV cm^{-1}) and three level of number of pulses (10, 20 and 30) were factorially combined in a triplicate experiment to evaluate the parameters' effect on the structural modification and surface hydrophobicity of the WPI.

3.3.4 Pulsed electric field (PEF) treatment

PEF treatments were performed using a high voltage generator TG2 (Food Process Engineering Laboratory, Macdonald Campus, McGill University) as described by Xiang et al. (2007). The simplified electric circuit of the pulse generator is shown in Figure 3.1. It consisted of a variable autotransformer (Powerstat Type 3PN116C) which supplied required voltage to the circuit. The voltage was elevated by high voltage transformer (62159A - Apotex Inc., Weston, ON, Canada) and then rectified by a high

voltage diode. The voltage across the treatment chamber depended on the distance between the spheres of the stainless steel, 15 mm diameter discharger. The break voltage for this diameter was calculated by the following equation (Armyanov et al., 2001).

$$V_o = 4.85\, h^{0.75} \quad (1)$$

where h is the distance between the spheres of the discharger (cm), V_o is the break voltage (kV).

The whey protein isolate (WPI) solution sample was placed in a parallel plate treatment chamber (TC-01001). The volume and the gap between the two electrodes of the treatment chamber were 4.2 ml and 0.7 cm, respectively. The voltage across the treatment chamber and current passing through it were monitored using a high voltage probe (P6015A, Tektronix, Inc., Beaverton, OR) and a current probe (P411, Pearson Electronics, Inc., Palo Alto, CA) connected to a two channel color digital oscilloscope (TDS3012, Tektronix Inc., Beaverton, OR). The WPI solution samples were treated by PEF with electric field intensities of 12, 16 and 20 kV cm^{-1}, capacitance of discharge capacitor 0.33 μF and number of pulses of 10, 20 and 30. This generator produced exponential decay pulse wave form and the exponential decay pulse frequency was 0.5 Hz. The sample temperature of the treatment chamber was maintained not higher than 35^{o}C by circulating 10^{o}C water from a water bath (W13, HAAKE Technik Gmbh, Germany) through the electrodes of the treatment chamber. The sample temperature was measured within 30 sec before and after each PEF treatment using a K type thermocouple (OMEGATM, Stamford, CT). The PEF-treated WPI solutions were studied immediately or within 2 hr.

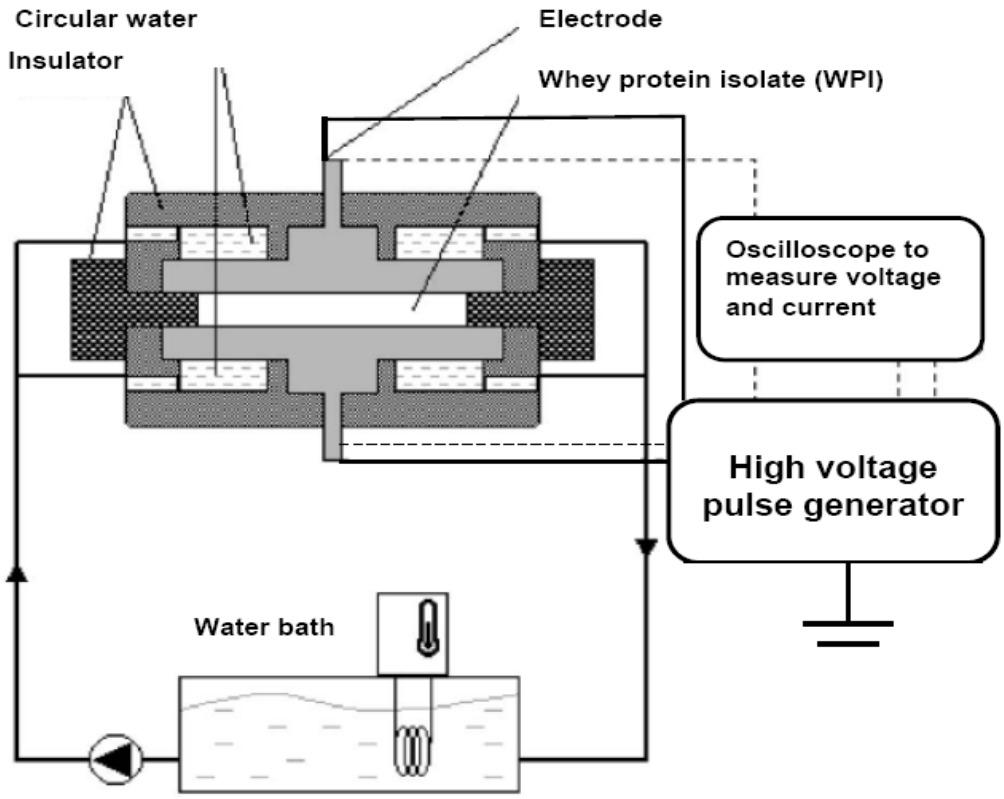

Figure 3. 1 Experimental set-up used for PEF treatments of whey protein isolate

3.3.5 Intrinsic fluorescence spectroscopy

The intrinsic tryptophan fluorescence of WPI was induced by using an excitation wavelength of 290 nm and the fluorescence emission spectrum monitored in the range of 305 to 400 nm (Kulmyrzaey & Defour, 2002; Garimella-Purna et al., 2005). The WPI solution sample should have an absorbance of ~0.1 at the excitation wavelength. This is because of the inner filter effect. This effect increases greatly with increasing absorbance of the sample, and can decrease the fluorescence intensity by either lowering the effective intensity of the exciting light or absorbing some of the fluorescence (Varley, 1994). The protein solution with low protein concentration and the fluorescence intensity was directly proportional to fluorophore concentration in the protein solution (Genot et al., 1992; Zhang et al., 2005). The fluorescence intensity was directly proportional to WPI protein concentration in the range from 0 to 0.05%; therefore each WPI solution sample

was diluted with distilled water (pH 7.0) to yield a final concentration of 0.03% before the fluorescence intensity measured. The tryptophan fluorescence intensity of WPI was measured at room temperature (25±1°C) using a Hitachi F-2000 fluorospectrophotometer (Hitachi, Ltd., Tokyo, Japan) with a slit for excitation of 2.0 nm and a scanning speed of 60 nm/min. Relative intrinsic fluorescence intensity (RIFI) (%) of WPI can be expressed by the following equation (2) and RIFI values (%) show the intrinsic fluorescence intensity changes of WPI with PEF treatments.

$$\text{RIFI\%} = \frac{\text{Intrinsic fluorescence intensity after PEF treatment}}{\text{Intrinsic fluorescence intensity before PEf treatment (control)}} \text{x100\%} \qquad (2)$$

3.3.6 Extrinsic fluorescence spectroscopy

The surface hydrophobicity of WPI was determined using an ANS fluorescence probe (Sklar et al., 1977; Hayakawa & Nakai, 1985; Bonomi & Iametti, 1991). For the measurement, 0.1 mL of ANS solution (5.0 mM in 0.05 M phosphate buffer, pH 7.0) was added to 9.9 mL of diluted WPI solution (0.03% protein concentration) and the mixture was left at room temperature (25 ± 1°C) for about 1 hr to ensure homogeneity. The WPI solution's extrinsic fluorescence intensity, using an excitation wavelength of 375 nm, was monitored over emission wavelengths of 420-560 nm, using a Hitachi F-2000 fluorospectrophotometer (Hitachi, Ltd., Tokyo, Japan). Relative extrinsic fluorescence intensity (REFI) (%) of WPI can be expressed by the following equation (3) and REFI values (%) show the extrinsic fluorescence intensity changes of WPI with PEF treatments.

$$\text{REFI\%} = \frac{\text{Extrinsic fluorescence intensity after PEF treatment}}{\text{Extrinsic fluorescence intensity before PEf treatment (control)}} \text{x100\%} \qquad (3)$$

3.4 STATISTICAL ANALYSIS

All experiments were conducted with triplicates; means and standard deviations were reported. Analysis of variance (ANOVA) was undertaken with PROC GLM in SAS software (SAS 9.1, SAS Institute Inc., Cary, NC, USA) for both intrinsic and extrinsic fluorescence intensity of PEF-treated WPI solutions. Differences between untreated 3% and 5% WPI controls and their respective individual electric field intensity × number of pulses treatment combinations were assessed using the Student's *t*-test. Significant differences and interactions between the factorially combined factors of WPI concentration, electric field intensity and number of pulses were assessed by ANOVA, using multiple comparisons by LSD.

Sigma Plot software (Version 11.0, Systat Software, Inc., Chicago, IL, USA) was used for regression analyses. Main effects were considered significant at the $P \leq 0.05$ level.

3.5 RESULTS AND DISCUSSION

3.5.1 Intrinsic fluorescence intensity of WPI

The tryptophan residues of proteins were selected as intrinsic fluorescent probes to monitor structural modification of whey protein with PEF treatments. Figure 3.2 illustrates the intrinsic tryptophan fluorescence emission spectra of 3% WPI with PEF treatments of 30 pulses and different electric field intensities (12, 16 and 20 kV cm^{-1}). The emission spectrum for the control sample showed the maximum fluorescence intensity at an emission wavelength of 333 nm. A PEF treatment of 16 kV cm^{-1} and 30 pulses resulted in a red shift of 2 nm (from 333 nm to 335 nm) in the emission wavelength, while a PEF treatment of 20 kV cm^{-1} and 30 pulses resulted in a red shift of 4 nm from 333 to 337 nm. The red shifts of 2-4 nm in emission spectra was significant ($P \leq 0.05$) although they were not easily noticeable in the emission spectra (Figure 3.2). The red shifts of the emission spectra observed with PEF treatments indicated that environments of tryptophan residues were modified. The intrinsic tryptophan

fluorescence intensities of WPI increased significantly ($P \leq 0.05$) with increasing electric field intensity ranging from 12, 16 and 20 kV cm^{-1}. The results of this study confirmed that whey protein unfolding with heat treatments caused a red shift of 2 ~ 10 nm) in the fluorescence spectrum due to exposure of the tryptophan residues to the aqueous solvent (Moro et al., 2001). Increases of the fluorescence intensity and red shifts observed after PEF treatments indicated changes in the polarity of tryptophan residues microenvironment in whey proteins from a less polar to a more polar environment. The results are in agreement with the report that high pressure treatment of α-lactalbumin at 400 MPa resulted in an increase in the intrinsic fluorescence intensity and red shifts (Tanaka et al., 1996). Heat-treated milk or ultra high temperature-treated milk had the red shifts of 5 ~ 8 nm (from 335 to 343 nm) in the maximum intrinsic tryptophan intensity because heat treatment caused partial denaturation of milk proteins and formation of fluorescent Maillard-reaction products. As a consequence of the denaturation, part of the tryptophan environments in proteins is modified (Kulmyrzaev & Dufour, 2002). Similar results were also reported for whey protein concentrate of 1% treated with high pressure treatments of 600 MPa, 50^{o}C, 0 to 30 min and the intrinsic fluorescence intensity of whey protein concentrate increased and a red shift of 2 ~ 4 nm appeared (Liu et al., 2005).

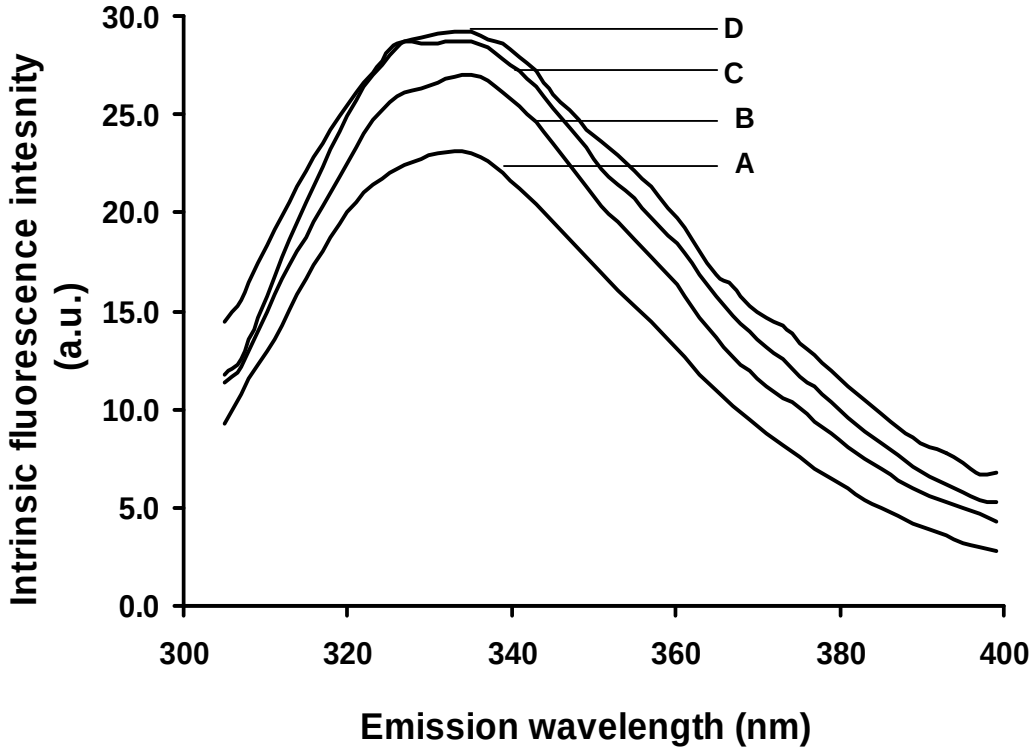

Figure 3. 2 Intrinsic fluorescence emission spectra of the control (no PEF treated) and PEF-treated (30 pulses) 3% WPI samples. The electric field intensity were: (A), Control; (B), 12 kV cm^{-1}; (C), 16 kV cm^{-1}; (D), 20 kV cm^{-1}, a.u. = arbitrary unit.

All PEF treatments (different electric field intensities and different number of pulses) significantly increased intrinsic fluorescence intensities of WPI solutions compared to the control samples (23.11 ± 0.15 a.u. and 25.57 ± 0.10 a.u. for 3 and 5% WPI, respectively). The change ranges of intrinsic fluorescence intensity of WPI at 12, 16, and 20 kV cm^{-1} were from 24.79 to 26.91 a.u., 25.44 to 28.73 a.u. and 25.96 to 29.16 a.u. for 3% WPI solution from 10 to 30 pulses, respectively. For 5% WPI solution, the change ranges of intrinsic fluorescence intensity at 12, 16, and 20 kV cm^{-1} were from 27.07 to 29.08 a.u., 27.42 to 29.08 a.u. and 28.56 to 30.76 a.u from 10 to 30 pulses, respectively as shown in Table 3. 1. The effects of PEF treatments on the intrinsic fluorescence intensities of 5% WPI were similar to those of 3% WPI. The increases in the intrinsic fluorescence intensities among control and PEF-treated 3% and 5% WPI were

significant ($P \leq 0.05$). The results indicated that electric field intensity, number of pulses and protein concentrations all increased the intrinsic fluorescence intensities of WPI.

Table 3. 1 Intrinsic fluorescence intensity changes of WPI with PEF treatments (intrinsic fluorescence intensity of WPI measured at the emission wavelength of 333 nm).

Protein concentration of WPI (%)	E (kV cm^{-1})	P (n)		
		10	20	30
3%	12	B 24.79 ± 0.09^{d*}	B 25.39 ± 0.59^{d}	A 26.91 ± 0.40^{d}
	16	C 25.44 ± 0.43^{cd}	B 26.74 ± 0.07^{c}	A 28.73 ± 0.03^{c}
	20	C 25.96 ± 0.43^{c}	B 27.24± 0.12^{bc}	A 29.16 ± 0.02^{bc}
5%	12	A 27.07 ± 0.36^{b}	A 27.82 ± 0.56^{ab}	A 29.08 ± 0.62^{bc}
	16	A 27.45 ± 0.53^{b}	A 28.19 ± 0.43^{ab}	A 29.76 ± 0.56^{b}
	20	B 28.56 ± 0.26^{a}	B 28.79 ± 0.36^{a}	A 30.76 ± 0.38^{a}

∗E = electric field intensity (kV cm^{-1}); P = number of pulses (n). Means with same letters in the column (a, b, c and d) are not significantly different ($P > 0.05$) and means with same letters in the row (A, B and C) are not significantly different ($P > 0.05$).

The RIFI values of WPI solution with PEF treatments are shown in Table 3. 2. The effects of PEF treatments on the RIFI values of 5% WPI were similar to the effects of PEF treatments on the RIFI values of 3% WPI. The higher protein concentration led to a higher electric conductivity or lower resistance than the lower protein concentration (Marcotte et al., 1998). Since 5% WPI showed low resistance and its pulse width was narrow, the treatment time for 5% WPI was shorter than that of 3% WPI. The RIFI values for 5% WPI were slightly lower than the EIFI values of 3% WPI. The RIFI value of 5% WPI was 120.30% at the PEF treatment of 20 kV cm^{-1} and 30 pulses, while the RIFI

value of 3% WPI was 126.18% under the same PEF conditions. For 3 and 5% WPI, increases in the RIFI were significantly different ($P \leq 0.05$) for different PEF treatments.

Table 3. 2 RIFI values (%) of WPI solutions with different PEF treatments.

Protein concentration of WPI (%)	E (kV cm^{-1})	P (n)		
		10	20	30
3%	12	B107.27±0.39^{c*}	B 109.87± 2.55^{bc}	A 116.4 ±1.73^{b}
	16	C110.08 ±1.86^{ab}	B 115.71 ±0.30^{a}	A 124.32 ± 0.13^{a}
	20	C112.33 ± 1.86^{a}	B 117.87± 0.52^{a}	A 126.18 ± 0.09^{a}
5%	12	B 105.87 ± 1.41^{c}	B 109.19 ± 1.64^{c}	A 113.73 ± 2.42^{b}
	16	B107.35 ± 2.07^{bc}	B 110.25 ± 1.68^{bc}	A 116.39 ± 2.19^{b}
	20	B 111.69 ± 1.02^{a}	B112.59 ± 1.41^{b}	A 120.30 ± 1.49^{b}

∗C = protein concentration of WPI solution (%); E = electric field intensity (kV cm^{-1}); P = number of pulses (n). Means with same letters in the column (a, b, c and d) are not significantly different ($P > 0.05$) and means with same letters in the row (A, B and C) are not significantly different ($P > 0.05$).

3.5.2 Extrinsic fluorescence intensity of WPI

The extrinsic fluorescence emission spectra of 3% WPI with different pulses (10, 20 and 30) at 20 kV cm^{-1} showed blue shifts of about 2-4 nm of the emission wavelength from 486 nm (the emission wavelength for the maximum fluorescence intensity for the control WPI) to 482-484 nm as shown in Figure 3.3. The extrinsic fluorescence intensities increased significantly ($P \leq 0.05$) with increasing number of pulses. Gatti et al. (1995) observed a similar phenomenon for micelle suspensions and attributed this shift to

a low-polarity environment with hydrophobic characteristics. These results indicated that the PEF-treated WPI bound more ANS in the less polar environment than did the native WPI. Similar observations were made by Yang et al. (2001) for β-lactoglobulin and Liu et al. (2005) for whey protein concentrate after high pressure treatments.

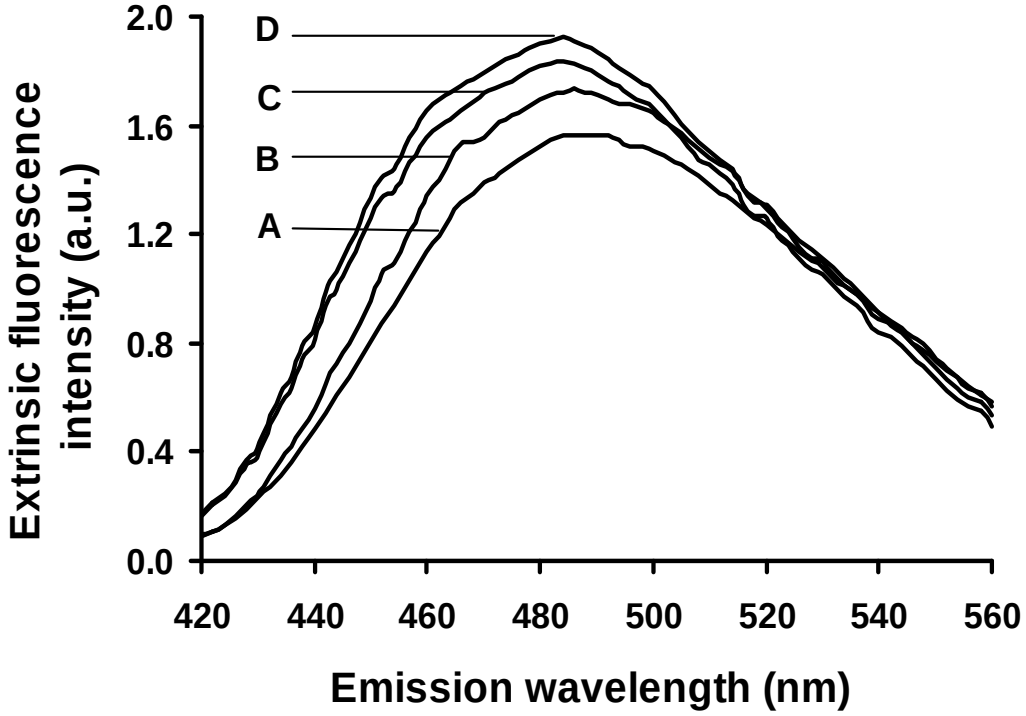

Figure 3. 3 Extrinsic fluorescence emission spectra of no treated control and PEF treated (20 kV cm^{-1}) 3% WPI samples. The number of pulses was: (A), Control (0 pulses); (B), 10 pulses; (C), 20 pulses and (D), 30 pulses.

For each WPI solution, all combinations of electric field intensity and number of pulses treatments yielded significantly ($P \leq 0.05$) greater extrinsic fluorescence intensities compared to control samples (1.57 ± 0.02 a.u. and 1.87 ± 0.04 a.u. for 3% and 5% WPI), respectively. The increase ranges of extrinsic fluorescence intensity of WPI at 12, 16, and 20 kV cm^{-1} were from 1.62 to 1.73 a.u., 1.65 to 1.79 a.u. and 1.74 to 1.91 a.u. for 3% WPI solution from 10 to 30 pulses, respectively. For 5% WPI solution, increase ranges of extrinsic fluorescence intensity at 12, 16, and 20 kV cm^{-1} were from 1.92 to 2.01 a.u.,

1.96 to 2.05 a.u. and 2.01 to 2.14 a.u from 10 to 30 pulses, respectively as shown in Table 3. 3. The effects of PEF treatments on the extrinsic fluorescence intensities of 5% WPI were similar to those of 3% WPI. The increases in the extrinsic fluorescence intensities among control and PEF-treated 3% and 5% WPI all were significant ($P \leq 0.05$). The results indicated that electric field intensity, number of pulses and protein concentrations all increased the extrinsic fluorescence intensities of WPI.

Table 3. 3 Extrinsic fluorescence intensity changes of WPI with PEF treatments (extrinsic fluorescence intensity of WPI measured at the emission wavelength of 486 nm).

Protein concentration of WPI (%)	E (kV cm^{-1})	P (n)		
		10	20	30
3%	12	B $1.62 \pm 0.01^{d*}$	A 1.70 ± 0.01^{d}	A 1.73 ± 0.04^{d}
	16	B 1.65 ± 0.04^{d}	AB 1.72 ± 0.02^{d}	A 1.79 ± 0.06^{d}
	20	C 1.74 ± 0.01^{c}	B 1.82 ± 0.01^{c}	A 1.91 ± 0.03^{c}
5%	12	B 1.92 ± 0.02^{b}	A 1.97 ± 0.02^{b}	A 2.01 ± 0.02^{b}
	16	B 1.96 ± 0.03^{b}	B 1.99 ± 0.01^{b}	A 2.05 ± 0.02^{b}
	20	B 2.01 ± 0.04^{a}	B 2.09 ± 0.02^{a}	A 2.14 ± 0.02^{a}

∗C = protein concentration of WPI solution (%); E = electric field intensity (kV cm^{-1}); P = number of pulses (n). Means with same letters in the column (a, b, c and d) are not significantly different ($P > 0.05$) and means with same letters in the row (A, B and C) are not significantly different ($P > 0.05$).

The REFI values of WPI solution with PEF treatments are shown in Table 3. 4. The effects of PEF treatments on the REFI values of 5% WPI were similar to those of 3% WPI. Since 5% WPI showed low resistance and its pulse width was narrow, the

treatment time for 5% WPI was shorter than that of 3% WPI. The REFI values for 5% WPI with PEF treatments were slightly lower than those of 3% WPI. The REFI value of 5% WPI was 114.6% at the PEF treatment of 20 kV cm^{-1} and 30 pulses, while the REFI value of 3% WPI was 121.9% under the same PEF conditions. The increase in the extrinsic fluorescence intensity was attributable to a higher number of hydrophobic groups binding to the ANS because of intermolecular interactions caused by PEF treatments (Hayakawa et al., 1992). The increase in extrinsic fluorescence intensity of WPI reflected the increase in the surface hydrophobicity with PEF treatments. WPI exposed to PEF treatments exhibited higher extrinsic fluorescence intensity than the native WPI. Jean et al. (2006) obtained similar results, finding that heat-induced whey protein aggregates were highly hydrophobic. This indicated that the structure of PEF-treated WPI became looser, thereby allowing ANS molecules to enter to the protein's hydrophobic core. The PEF-treated WPI changed its three-dimensional structure so as to expose small hydrophobic pockets previously inaccessible to solvent (Alvarez, 2005).

Table 3. 4 REFI values (%) of WPI solutions with different PEF treatments.

Protein concentration of WPI (%)	E (kV cm^{-1})	P (n)		
		10	20	30
3%	12	B $103.4 \pm 0.6^{c*}$	A 108.5 ± 0.6^{c}	A 110.4 ± 2.6^{c}
	16	C 105.3 ± 2.6^{bc}	AB 109.8 ± 1.3^{c}	A 114.3 ± 3.8^{b}
	20	C 111.1 ± 0.6^{a}	B 116.2 ± 0.6^{a}	A 121.9 ± 1.9^{a}
5%	12	B 102.9 ± 1.1^{c}	A 105.5 ± 1.1^{d}	A 107.1 ± 0.5^{c}
	16	B 105.0 ± 1.6^{bc}	B 106.6 ± 0.5^{d}	A 109.8 ± 1.1^{c}
	20	B 107.7 ± 2.1^{b}	A 112.0 ± 1.1^{b}	A 114.6 ± 1.1^{b}

*C = concentration of whey protein isolate, E = electric field intensity (kV cm^{-1}), and P = number of pulses (n). Means with same letters in the column (a, b, c and d) are not significantly different ($P > 0.05$) and means with same letters in the row (A, B and C) are not significantly different ($P > 0.05$).

3.6 CONCLUSION

PEF treatments with different electric field intensities and pulses affected the intrinsic and extrinsic fluorescence intensities of WPI solutions. For both 3% and 5% WPI, there were increases in the intrinsic fluorescence intensities and red shifts at the emission wavelength when the electric field intensities and number of pulses increased. The extrinsic fluorescence intensity also increased with increasing electric field intensities and number of pulses but resulted in blue shifts at the emission wavelength. The changes in the fluorescence intensities for 3% WPI were similar to those of 5% WPI. Overall, the results indicated that electric field intensity, number of pulses and protein concentrations modified protein structure of WPI exposed to PEF. The results indicated

that PEF treatments increased the extrinsic fluorescence intensity and also increased the surface hydrophobicity of WPI.

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CONNECTING TEXT

A comprehensive review of literature demonstrated the need for a study regarding the effects of pulsed electric field (PEF) on the structural modification and thermal property of a food protein by using differential scanning calorimetery (DSC) and fluorescence spectroscopy techniques. The following study sought to determine the structural modification and thermal property of whey protein isolate (WPI). The structural modification, surface hydrophobicity and thermal properties of whey proteins with PEF treatments were measured by fluorescence spectroscopy and DSC techniques.

Chapter 4 describes how the structural modification, surface hydrophobicity and thermal properties of whey protein isolate with PEF treatments were evaluated using fluorescence spectroscopy and DSC techniques. PEF treatments included different electric field intensities of 12, 16 and 20 kV cm^{-1} and different pulses of 10, 20 and 30. The results of this study confirmed that structural modification, and changes in surface hydrophobicity and thermal properties of whey protein isolate occurred through PEF treatments. They also confirmed that PEF treatments denatured protein structure and increased surface hydrophobicity and changed the thermal properties of whey protein isolate.

4. MODIFICATION OF WHEY PROTEIN ISOLATE HYDROPHOBICITY AND THERMAL PROPERTIES BY PULSED ELECTRIC FIELDS

4.1 ABSTRACT

This study evaluated the structural modification, surface hydrophobicity and thermal properties of whey protein isolate (WPI) treated by pulsed electric fields (PEF). Structural modification and thermal properties of WPI were evaluated using fluorescence spectroscopy and differential scanning calorimetry (DSC) techniques. The effects of PEF treatments (12, 16 and 20 kV cm^{-1}, number of pulses ranging from 10-30) on intrinsic fluorescence intensity of WPI and extrinsic 1-anilino-naphthalene-8-sulfonate (ANS) fluorescence intensity of WPI were studied. PEF treatments of WPI resulted in an increase in the intrinsic tryptophan fluorescence intensity and 2-4 nm red shifts, which indicated changes in the polarity of tryptophan residues' microenvironment in whey proteins from less polar to more polar. There was a 23% increase in the relative fluorescence intensity of WPI after PEF treatments of 20 kV/cm and 30 pulses. PEF treatments of WPI resulted in an increase in extrinsic fluorescence intensity indicating an increase in the surface hydrophobicity of WPI. An increase in relative fluorescence intensity of WPI indicated an increase in surface hydrophobicity, which is positively correlated with functional properties. The apparent enthalpy and denaturation temperatures of WPI samples were modified by PEF treatments. The remaining native whey protein was about 56% by a PEF treatment of 20 kV cm^{-1} and 30 pulses. PEF treatments affected structural modification, altered surface hydrophobicity and thermal properties of whey protein isolate (WPI).

Keywords: Pulsed electric fields (PEF), whey protein isolate (WPI), relative fluorescence intensity, hydrophobicity, differential scanning calorimetry (DSC), protein structure, thermal property.

4.2 INTRODUCTION

Whey protein isolate (WPI) is the purest whey protein and contains more than 90% β-lactoglobulin and α-lactalbumin. It is an excellent source of high amino acids and an important source of functional ingredients used in many formulated foods, such as processed meat, as well as bakery and dairy products (Kinsella & Whitehead, 1989). Whey proteins possess varying functional properties because of their dynamic structures and amphiphilic nature. The dynamic structure and flexibility of whey proteins indicate the possibility of further enhancing their functional properties, such as those of foaming and emulsifying. The functional properties of whey proteins are influenced by their structural modification or denaturation (Kinsella et al., 1994). The protein denaturation may be affected by many factors including heat treatment, pH, ionic strength and solvent system, high pressure and pulsed electric fields (PEF) treatments (Rhim et al., 1990; Phillips et al., 1991a; Phillips et al., 1991b; Boye et al., 1995; Hayakawa et al., 1992; Hinrichsa & Rademacher, 2005; Barsotti et al., 2002; Perez & Pilosof, 2004). To obtain desired functional properties from a protein, the special operating conditions are required to cause protein denaturation or protein structural modification. Measurements of structural modification and thermal properties are very useful in predicting the extent of whey protein denaturation and resulting functional properties.

PEF processing presents unique advantages over conventional thermal processing methods. PEF-treated foods are microbiologically safe, minimally processed, nutritious and freshlike (Qin et al., 1996). Most studies have been carried out on the effects of PEF on the structural modification and inactivation of enzymes (Ho et al., 1997; Loey et al., 2002; Espachs-Barroso et al., 2003; Zhong et al., 2005a; Zhong et al., 2005b; Zhang et al., 2006; Zhang et al., 2007). The available literature regarding whey protein structural modification and thermal properties induced by PEF treatment is limited (Fernández-Díaz et al., 2000; Perez & Pilosof, 2004). The current work was undertaken to evaluate the effects of PEF on structural modification and resultant thermal properties of WPI.

Fluorescence spectroscopy is a sensitive, rapid, and non-invasive analytical method that provides information on the presence of fluorescent molecules and their

environment in biological and food samples (Herbert et al., 1999; Christensen et al., 2003). Fluorescence properties of aromatic amino acids of proteins have been used to study protein structure or protein-hydrophobic molecule interactions (Defour et al., 1994; Defour & Riaublanc, 1997). Differential scanning calorimetry (DSC) is a powerful technique to study the thermodynamics of protein stability that can provide a basic understanding of protein structural modification (Thorarinsdottir et al., 2002; Kurganov et al., 1997).

The objective of this study was to investigate the effects of PEF treatments on structural modification, surface hydrophobicity and thermal properties of WPI using fluorescence spectroscopy and DSC techniques.

4.3 MATERIALS AND METHODS

4.3.1 Materials

BiPRO whey protein isolate (WPI) powder (lot no. JE348-6-440) was provided by Davisco Foods International, Inc. (Le Sueur, MN). The products contained 93% protein, 0.2% fat, 2% ash, 4.8% moisture (measured by the standard proximate analysis procedures, company offered this information, did not carry out analysis again). The pH of 10% WPI solution at 20^{o}C was 7.1. The fluorescence spectroscopy probe, 1-anilino-8-naphthalenesulfonate (ANS) was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). All of the chemicals (such as hydrochloric acid and buffer solutions) used were purchased from Fisher Chemicals (Fairlawn, NJ).

4.3.2 Preparation of samples

Three percent WPI solutions were prepared using distilled water. The pH of the WPI solution was adjusted to 7.0 using 1M hydrochloric acid (HCl). The prepared WPI solution was allowed to wait for about 2 hr to ensure homogeneity prior to PEF treatments.

4.3.3 Pulsed electric field treatments

PEF treatments were performed using a high voltage generator TG2 as described in Chapter 3. The generator produced exponential decay pulses with pulse frequency of 0.5 Hz. The capacitance of the discharge capacitor of the generator was 0.33 μF. The structural modification and thermal properties of WPI were investigated under PEF conditions of three electric field intensities (12, 16 and 20 kV cm^{-1}) and three different pulses (10, 20 and 30).

4.3.4 Intrinsic fluorescence measurements

The intrinsic tryptophan fluorescence intensity of the WPI was measured at room temperature (25±1.0^{o}C), using an excitation wavelength of 290 nm. The emission spectra was monitored at emission wavelengths ranging from 305 to 400 nm using a Hitachi F-2000 fluorospectrophotometer (Hitachi, Ltd., Tokyo, Japan) with an excitation slit of 2.0 nm and a scanning speed of 60 nm/min (Kulmyrzaey & Defour, 2002). Diluted whey protein solutions (0.03%) were used in fluorescence measurements. The absorbance of the diluted samples was maintained below 0.1 in order to avoid a screening effect leading to a decrease in the fluorescence intensity and a distortion of the excitation spectra. High absorption of the sample can result in the distortion of the incident beam and may result in the reabsorption of the emitted light (Genot et al., 1992; Sharma & Schulman, 1999; Zhang et al., 2005).

4.3.5 Surface hydrophobicity of WPI using ANS probe

Surface hydrophobicity of WPI solutions was measured according to Kato & Nakai (1980) and Nakai (1983) using the fluorescence probe ANS. The PEF-treated WPI solutions and the control were diluted by using distilled water to a final protein concentration of 0.03%. Then 0.1 mL of ANS (50 mM in 0.05 M phosphate buffer, pH 7.0) was added to 9.9 mL of the diluted WPI sample and mixed. Extrinsic fluorescence intensity of WPI was assayed at an excitation wavelength of 375 nm and an emission

wavelength ranging from 420-560 nm using the same fluorospectrophotometer. Surface hydrophobicity in WPI samples was defined as the ratio of the extrinsic fluorescence intensity to the whey protein concentration (0.03%), as described by Li et al. (2007).

4.3.6 Differential scanning calorimetry (DSC) measurement

The thermal properties of whey protein in WPI solutions were examined using a differential scanning calorimeter (Q100, TA Instruments, New Castle, DE, USA). The DSC was calibrated using an indium standard. Ten mg of WPI solution was accurately weighed and put in the aluminum pan. The pan was hermetically sealed. WPI samples were equilibrated at 20^{o}C for 2 hr. The temperature was then increased from 20 to 90°C at a controlled heating rate of 5°C/min and the samples were scanned. An empty pan was used in the treatment cell of the DSC as a reference for each run. All DSC measurements were carried out in triplicate.

The denaturation temperatures (T_d) and the apparent enthalpy (ΔH) of WPI were computed from each thermogram by the TA instruments software (Universal Analysis, TA version 4.10). Enthalpy values were based on the total weight of WPI. In order to calculate the amounts of the native protein of WPI after each PEF treatment, the following equation was used:

$$\% \text{ Native protein} = (\Delta H_2/\Delta H_1) \text{ x}100\% \quad (1)$$

where ΔH_1 is the enthalpy of the untreated WPI, ΔH_2 is the enthalpy corresponding to the PEF-treated WPI. Data reported represent the average and standard deviation of three replicates of the PEF-treatments.

4.4 STATISTICAL ANALYSIS

All experiments were conducted with triplicates; means and standard deviations were reported. Analysis of variance (ANOVA) was undertaken with SAS Software (SAS

9.1, SAS Institute Inc., Cary, NC, USA) for all data analysis. Main effects were considered significant when $P \leq 0.05$ level.

4.5 RESULTS AND DISCUSSION

4.5.1 Intrinsic fluorescence intensity of WPI

Intrinsic tryptophan fluorescence emission spectra of WPI solutions with PEF treatments of 20 kV cm^{-1} and 10, 20 and 30 pulses are shown in Figure 4.1. The emission spectrum for the control sample showed the maximum fluorescence intensity at an emission wavelength of 333 nm, indicating the characteristic peak of tryptophan residue in the fluorescence emission spectra. Increases in the intrinsic fluorescence intensity of WPI and red shifts of the emission wavelength were observed after PEF treatments. A PEF treatment of 20 kV cm^{-1} and 20 pulses resulted in a 2 nm red shift (333 nm to 335 nm) in the emission wavelength. There was a sharp increase in intrinsic fluorescence intensity and resulting in a 4 nm red shift (333 nm to 337 nm) in the emission wavelength for a PEF treatment of 20 kV cm^{-1} and 30 pulses though this was not easy to observe in the emission spectra. This result was similar to the results with 1% whey protein concentrate under high pressure treatments of 600 MPa, 50^{o}C, 0 to 30 min reported by Liu et al. (2005). Whey protein unfolding caused a red shift in the fluorescence emission spectrum due to exposure of the tryptophan residues to the aqueous solvent (Moro et al., 2001). Increases in the intrinsic fluorescence intensity and red shifts observed after PEF treatments indicated changes in the polarity of tryptophan residues' microenvironment of whey proteins from a less polar to a more polar environment.

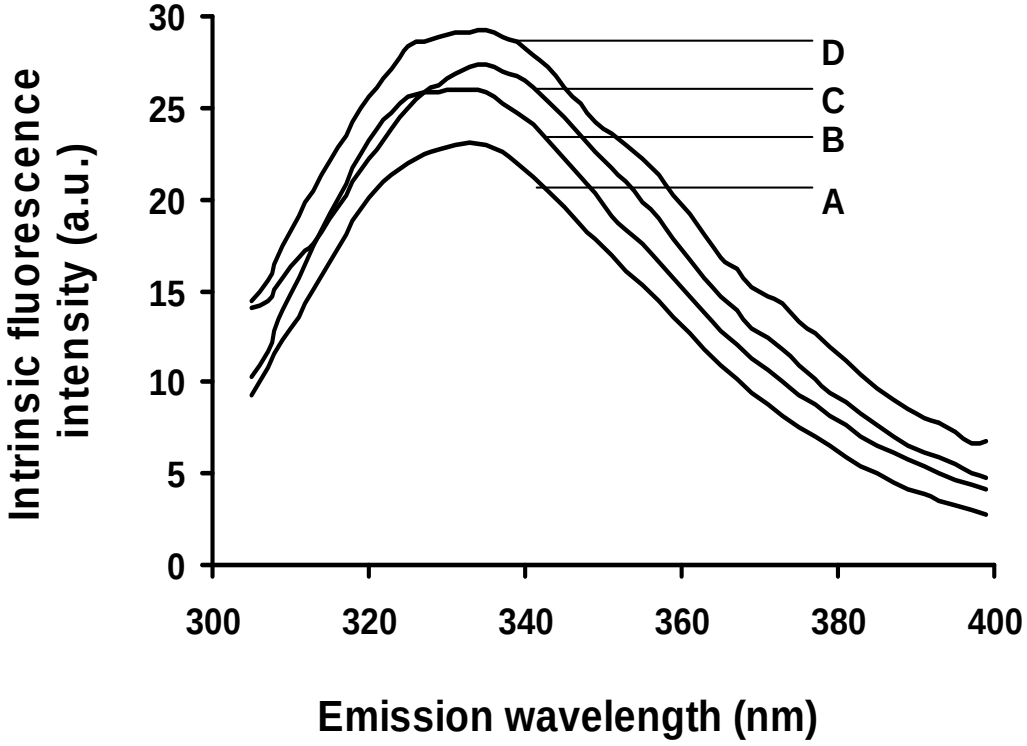

Figure 4. 1 Intrinsic tryptophan emission spectra of non-treated control and PEF treated WPI samples (20 kV cm^{-1}). The number of pulses was: (A) Control (0 pulse); (B) 10 pulses; (C) 20 pulses; (D) 30 pulses.

PEF treatments of the electric field intensity (12, 16 and 20 kV cm^{-1}) and the number of pulses (10, 20 and 30) affected the intrinsic fluorescence intensity of WPI solution as shown in Table 4.1. All PEF treatments resulted in significant ($P \leq 0.05$) increases in the intrinsic fluorescence intensity of WPI solutions compared to the control (23.11 ± 0.15 a.u.). The intrinsic tryptophan fluorescence intensities of WPI at 12, 16, and 20 kV cm^{-1} increased from 24.79 to 26.91 a.u., 25.44 to 28.73 a.u. and 25.96 to 29.16 a.u. for the number of pulses of 10 and 30, respectively. The results indicated that electric field intensity and number of pulses both increased the intrinsic fluorescence intensities of WPI. The results obtained in this study were consistent with other studies reported by Zhong et al. (2005b) and Zhang et al. (2007). In another study, PEF treatments resulted in an increase in the intrinsic fluorescence intensity of horseradish

peroxidase attributed to modification of the tertiary structure of the horseradish peroxidase (Zhong et al., 2005b). The increase in the intrinsic fluorescence intensity of WPI at the wavelength of 333 nm reflected the alteration of the tertiary structure of WPI.

Table 4. 1 Electric field intensity and number of pulses effects on the intrinsic fluorescence intensity of WPI (at the emission wavelength of 333 nm)

Electric field intensity ($kV\ cm^{-1}$)	Number of pulses (n)		
	10	20	30
12	B $24.79 \pm 0.09^{b*}$	B 25.39 ± 0.59^{b}	A 26.91 ± 0.40^{b}
16	C 25.44 ± 0.43^{ab}	B 26.74 ± 0.07^{a}	A 28.73 ± 0.03^{a}
20	C 25.96 ± 0.43^{a}	B 27.24 ± 0.12^{a}	A 29.16 ± 0.02^{a}

*****Means with same letters in the column (a and b) are not significantly different ($P > 0.05$) and means with same letters in the row (A, B and C) are not significantly different ($P > 0.05$).

4.5.2 Surface hydrophobicity increase of WPI with PEF treatments

For 3% WPI solution, combinations of electric field intensity and number of pulses treatments yielded significantly ($P \leq 0.05$) greater extrinsic fluorescence intensities compared to control samples (1.57 ± 0.02 a.u) as shown in Table 4. 2. The increase ranges of extrinsic fluorescence intensity of WPI at 12, 16, and 20 $kV\ cm^{-1}$ were from 1.62 to 1.73 a.u., 1.65 to 1.79 a.u. and 1.74 to 1.91 a.u. for 3% WPI solution from 10 to 30 pulses, respectively. The increases in the extrinsic fluorescence intensities between control and PEF-treated 3% WPI were significant ($P \leq 0.05$). The results indicated that electric field intensity and number of pulses increased the extrinsic fluorescence intensities of WPI.

Table 4. 2 Electric field intensity and number of pulses effects on the extrinsic fluorescence intensity of WPI (at the emission wavelength of 486 nm).

Electric field intensity	P (n)		
(kV cm^{-1})	10	20	30
12	B $1.62 \pm 0.01^{b*}$	A 1.70 ± 0.01^{b}	A 1.73 ± 0.04^{b}
16	B 1.65 ± 0.04^{b}	AB 1.72 ± 0.02^{b}	A 1.79 ± 0.06^{b}
20	C 1.74 ± 0.01^{a}	B 1.82 ± 0.01^{a}	A 1.91 ± 0.03^{a}

*****Means with same letters in the column (a and b) are not significantly different ($P > 0.05$) and means with same letters in the row (A, B and C) are not significantly different ($P > 0.05$).

Surface hydrophobicity of proteins is one of the structural characteristics used to evaluate the change in protein structure (Nakai, 1983). Increases in surface hydrophobicity were reflected in the extrinsic fluorescence spectra in the presence of WPI treated by PEF at 16 kV cm^{-1} with 10, 20 or 30 pulses. Figure 4.2 shows that surface hydrophobicity increased with increasing number of pulses from 10 to 30. In particular, for a PEF treatment of 16 kV cm^{-1} and 30 pulses, surface hydrophobicity of WPI was 114.3% of the control. Increases in the surface hydrophobicity of WPI with PEF treatments of 30 pulses and different electric field intensities of 12, 16 and 20 kV cm^{-1} are shown in Figure 4.3. A significance increase ($P \leq 0.05$) in surface hydrophobicity was observed with increasing the electric field intensity. The surface hydrophobicity of WPI at 20 kV cm^{-1} and 30 pulses was 121.9% of the control. The results were similar to the results of Li et al. (2007) with PEF treatment at 30 kV cm^{-1} and treatment time of 288 μs with soy protein isolate, where its surface hydrophobicity was 120% of the control.

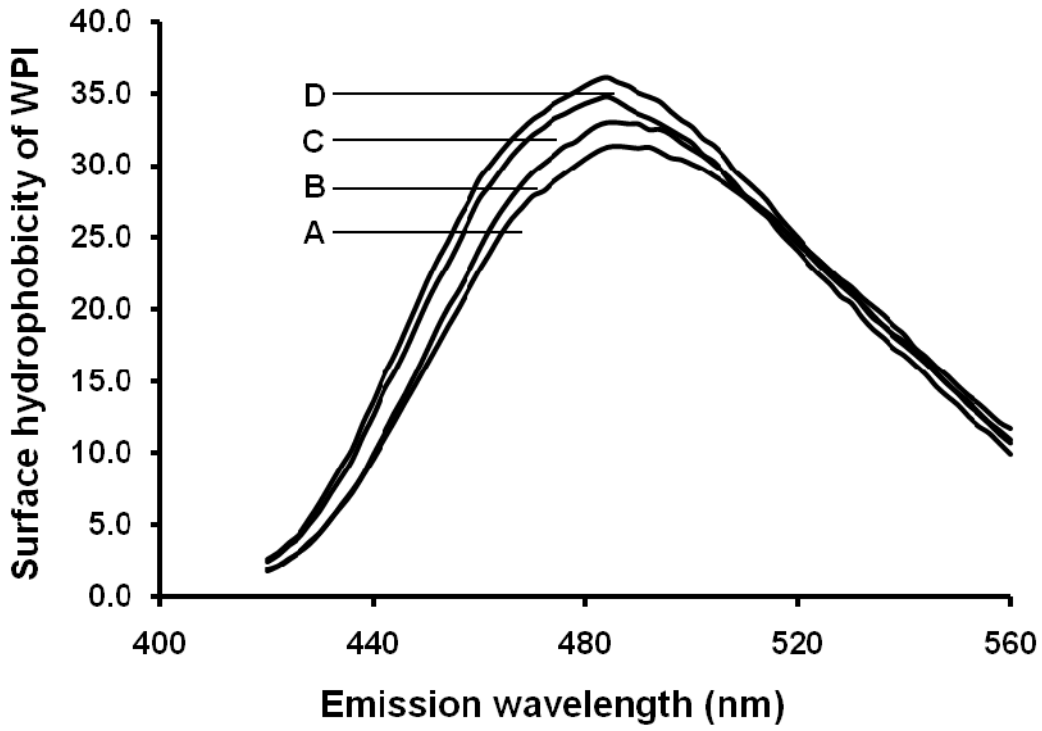

Figure 4. 2 Surface hydrophobicity of non treated control and PEF treated WPI samples (16 kV cm^{-1}). The number of pulses was: (A), Control; (B), 10 pulses; (C), 20 pulses and (D), 30 pulses.

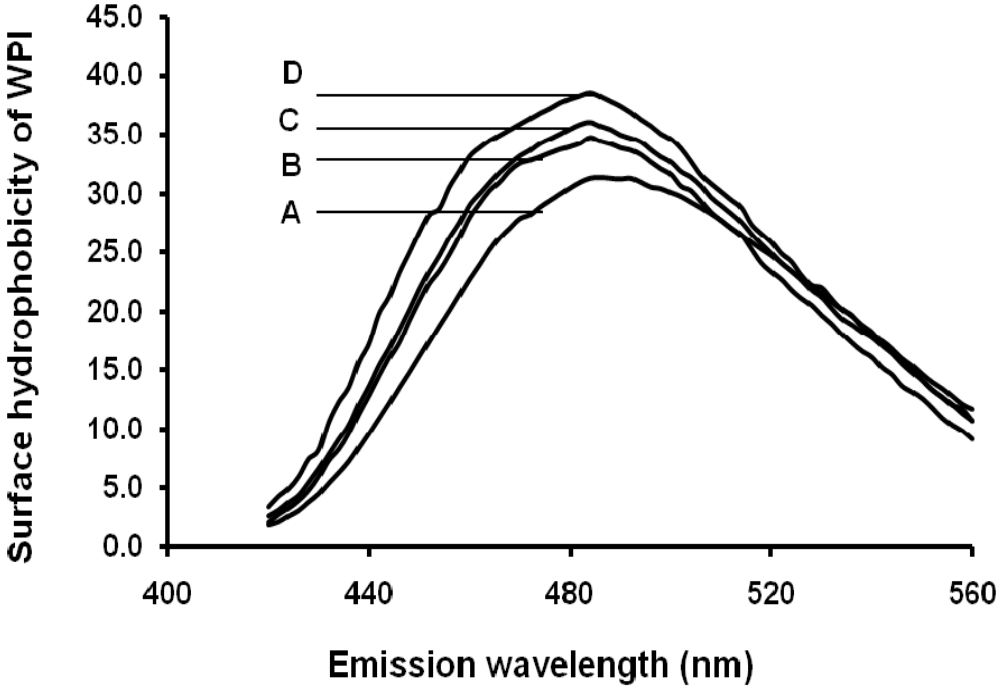

Figure 4. 3 Surface hydrophobicity of non treated control and PEF treated WPI samples (30 pulses). The electric field intensities were: (A), Control; (B), 12 kV cm^{-1}; (C), 16 kV cm^{-1} and (D), 20 kV cm^{-1}.

These results indicated that a higher number of pulses or a higher electric field intensity induced a greater molecular unfolding of WPI, destroying hydrophobic interactions of protein molecules, making structural modification of whey protein, causing more hydrophobic groups and regions inside the molecules to be exposed to the outside, thus increasing the surface hydrophobicity of WPI. The results were similar to those for heat-treated whey protein aggregates (Jean et al., 2006), and high pressure-treated whey protein (Lee et al., 2006), which both showed a significant rise in surface hydrophobicity, due to protein unfolding. These results indicated that PEF-treated WPI protein structure became loose, thereby allowing ANS molecules to reach the hydrophobic core of the protein. This increase in surface hydrophobicity of β-lactoglobulin heated at temperatures ranging from 60 to 85°C since increasing heat-

treatment was related to the heat-induced irreversible unfolding of the protein (Relkin, 1998). Alvarez (2005) also obtained similar results with high pressure treatments of 400 to 500 MPa, where whey protein concentrate changed its three-dimensional structure to expose small hydrophobic pockets previously inaccessible to solvents.

4.5.3 Thermal properties of WPI with PEF treatment

DSC thermograms of the control and the PEF-treated WPI at the electric field intensity of 20 kV cm^{-1} and 30 pulses are shown in Figure 4.4. Both the denaturation temperatures (T_d) and the apparent enthalpy (ΔH) were modified by PEF treatments. Changes of the apparent enthalpy corresponded to dimmer dissociation and denaturation processes (endothermic) which were superimposed to an aggregation process (exothermic) during the time scale of DSC measurements (Relkin et al., 1998). The DSC thermogram of the control sample had two peak denaturation temperatures namely ~68 and ~78^{o}C. Denaturation temperatures (T_{d1} and T_{d2}) of WPI involved in the denaturation processes were changed by PEF treatment at 20 kV cm^{-1} and 30 pulses.

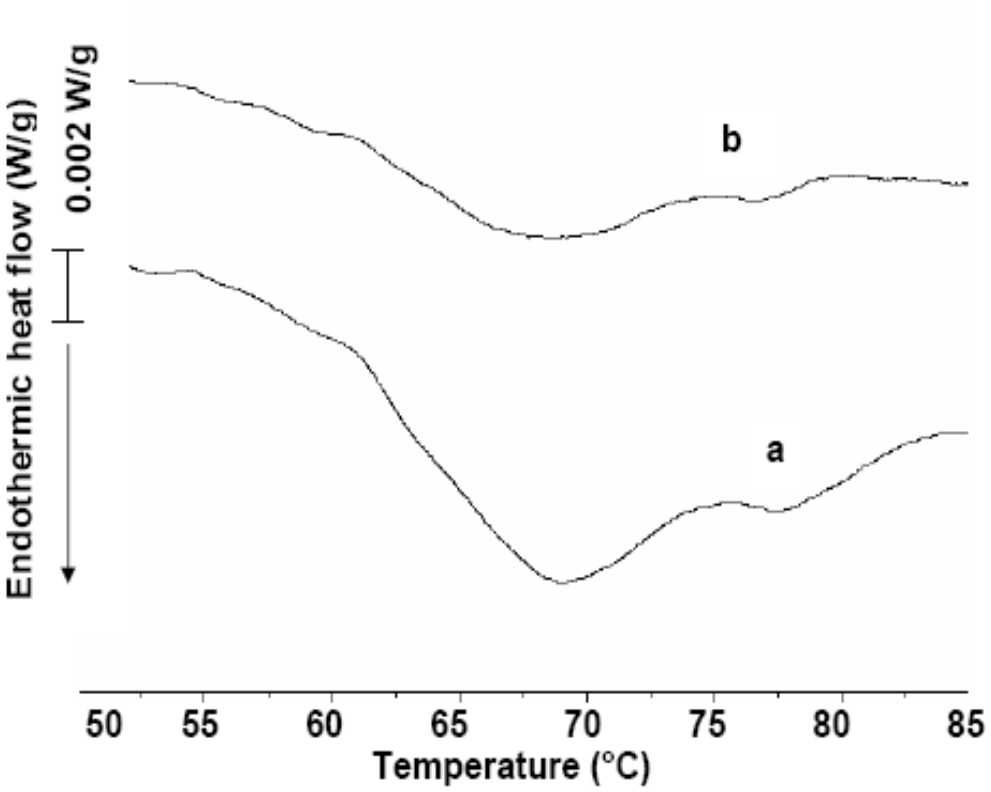

Figure 4. 4 DSC thermograms of the non treated control and PEF treated WPI samples. (a), Control; (b), PEF condition of electric field intensity of 20 kV cm^{-1} and 30 pulses.

PEF treatments of electric field intensity (12, 16 and 20 kV cm^{-1}), the number of pulses (10, 20 and 30) affected the denaturation temperatures (T_{d1} and T_{d2}) of WPI solution as shown in Tables 4.3 and 4.4. The number of pulses and electric field intensity had significant ($P \leq 0.05$) effect on T_{d1} (Table 4.3), and on T_{d2} (Table 4.4). The results indicated that electric field intensity and number of pulses both altered the denaturation temperatures when WPI treated by PEF. The results were similar to other results of β-lactogalobulin's denaturation temperature alteration with PEF treatments of 12.5 kV cm^{-1} and different number of 1-10 pulses (Perez & Pilosof, 2004).

Table 4. 3 Electric field intensity and number of pulses effects on denaturation temperature T_{d1} of WPI sample.

Electric field intensity (KV cm^{-1})	Number of pulses (n)		
	10	20	30
12	B 66.29^{b}*	B 66.17^{b}	A 67.65^{a}
16	AB 66.77^{ab}	A 66.56^{b}	B 66.94^{a}
20	B 66.76^{b}	AB 66.43^{c}	A 67.49^{a}

*Values with same letters in the column (a, b and c) are not significantly different ($P>$ 0.05) and means with same letters in the row (A and BC) are not significantly different ($P>0.05$), standard deviation for each value was not shown here.

Table 4. 4 Electric field intensity and number of pulses effects on denaturation temperature (T_{d2}) of WPI sample.

Electric field intensity (kV cm^{-1})	Number of pulses (n)		
	10	20	30
12	B 77.16^{b}*	B 77.09^{b}	B 78.13^{a}
16	A 77.90^{b}	A 77.57^{c}	A 78.67^{a}
20	B 77.23^{b}	B 77.19^{b}	B 78.20^{a}

*****Values with same letters in the row (a, b and c) are not significantly different ($P>0.05$) and means with same letters in the column (A and B) are not significantly different ($P >$ 0.05), standard deviation for each value was not shown here.

PEF treatments of electric field intensity (12, 16 and 20 kV cm^{-1}) and number of pulses (10, 20 and 30) affected the enthalpy value of WPI solution in a highly significant manner ($P \leq 0.05$, Table 4.5). The residual denaturation enthalpy provided a net value

from a combination of endothermic reactions like the disruption of hydrogen bonds, and exothermic processes, including the break-up of hydrophobic interactions and protein aggregation reported by Ma & Harwalkar (1991). An increase in the electric field intensity led to a significant decrease in the apparent enthalpy, while modifying the protein structure of WPI. The remaining native whey protein was approximately 59% after a PEF treatment of 16 kV cm^{-1} and 30 pulses, while the remaining native whey protein was approximately 62% after a PEF treatment at 12 kV cm^{-1} and 30 pulses. Perez & Pilosof (2004) reported that PEF treatment of 12.5 kV cm^{-1} and capacitance of 40 μF changed the thermal stability of β-lactoglobulin by shifting the temperature by 4~5^{o}C and leaving 60% remaining native protein after 10 pulses. As a result, DSC thermograms of the PEF-treated WPI exhibited thermal denaturation of whey protein. The results were similar to those from other researchers (Hayakawa et al., 1996; Van der Planchen et al., 2007) who explained that a reduction in residual enthalpy indicated a partial loss of protein structure after high pressure treatments.

Table 4. 5 Electric field intensity and number of pulses effects on enthalpy ΔH (J g^{-1}) for WPI solution.

Electric field intensity (kV cm^{-1})	Number of pulses (n)		
	10	20	30
12	A 0.056^{a}*	A 0.0486^{b}	A 0.044^{c}
16	B 0.053^{a}	B 0.0466^{b}	B 0.0423^{c}
20	C 0.0493^{a}	C 0.0463^{b}	C 0.0403^{c}

*****Values with same letters in the row (a, b and c) are not significantly different ($P > 0.05$) and means with same letters in the column (A, B and C) are not significantly different ($P > 0.05$), standard deviation for each value was not shown here.

4.6 CONCLUSION

The effects of PEF treatments on structural modification and thermal properties of WPI were studied. The increases in the intrinsic tryptophan fluorescence intensity of WPI and red shifts of 2-4 nm of the emission wavelength of the maximum intrinsic fluorescence intensity were observed following PEF treatments. Extrinsic fluorescence intensity changes of WPI were related to the structural modification of WPI. This confirmed that the main parameters influencing structural modification of WPI were the electric field intensity and number of pulses. Comparison of DSC thermal parameters of whey protein showed that the thermal stability of whey protein was greatly reduced by PEF treatments. The remaining native whey protein was about 56% after PEF treatment at 20 kV cm^{-1} and 30 pulses. In the electric field intensity, the increase in the number of pulses also increased protein denaturation of WPI. Further studies on the effect of PEF on the structural modification of whey protein may be needed to understand its modification mechanism.

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CONNECTING TEXT

A comprehensive review of literature demonstrated the need for a study regarding the effects of pulsed electric fields (PEF) on structural modification of food proteins by using fluorescence spectroscopy. The results were different with PEF treatments for different food proteins. The following study sought to determine the structural modification and surface hydrophobicity of soy protein. The structural modification of soy protein with PEF treatments was measured by fluorescence spectroscopy.

Chapter 5 describes that the structural modification and surface hydrophobicity of soy protein isolate (SPI) with PEF treatments were evaluated using fluorescence spectroscopy. PEF treatments used different electric field intensities of 22 and 25 kV cm^{-1} and number of pulses of 30, 60, 90 and 120. The results of this study confirmed that structural modification and an increase in surface hydrophibicity of soy protein had resulted from PEF treatments. The results confirmed that PEF treatments partially denatured the protein structure and increased their surface hydrophobicity.

5. THE PULSED ELECTRIC FIELD-INDUCED STRUCTURAL MODIFICATION OF SOY PROTEIN AS STUDIED BY FLUORESCENCE SPECTROSCOPY

5.1 ABSTRACT

This study evaluated structural modification and surface hydrophobicity of soy protein isolate (SPI) treated with pulsed electric fields (PEF). Fluorescence spectroscopy technique was employed to monitor the effects of PEF on the structural modification of soy protein. The effects of PEF treatments (electric field intensity of 22 and 25 kV cm^{-1} and number of pulses of 30, 60, 90 and 120) on intrinsic fluorescence intensity and surface hydrophobicity of soy protein were studied. PEF treatments of SPI increased the intrinsic tryptophan fluorescence intensity and led to 2-6 nm red shifts of the emission wavelengths, which indicated that the microenvironment of soy protein's tryptophan residues changed from a less polar to a more polar environment. The extrinsic fluorescence intensity of soy protein also showed an increasing trend and 2-6 nm blue shifts, which indicated soy protein fractions were partially denatured under PEF treatments, leading to an increase in their surface hydrophobicity. These results indicated that PEF treatments indeed modified protein structure of SPI and increased its surface hydrophobicity.

Keywords: Pulsed electric fields (PEF), soy protein isolate (SPI), protein structure, tryptophan and surface hydrophobicity.

5.2 INTRODUCTION

Soybean protein isolates (SPI) are prepared from defatted soybean flours by water extraction under mildly alkaline condition. SPI contains important vegetable proteins, particularly 7S (β-conglycinin) and 11S (glycinin) globulins, so named on the basis of

sedimentation rates. It is commonly used as functional ingredients for food formulation because it has excellent functional properties and good nutritional values. Different food processing treatments affect structural and physicochemical properties of SPI (Petruccelli & Anon, 1994; Puppo et al., 1995; Puppo & Anon, 1999). It is well known that heat treatments induce dissociation, denaturation, and aggregation of soy protein's subunits (Yamauchi et al., 1991; Sorgentini et al., 1995). However, heat treatments for soy protein and soy products have negative effects on their solubility and water absorption characteristics (Kinsella, 1979), and mildly heat-treated products produce strong off-flavors, which is the primary problem for developing soy protein foods (Fukushima, 2000). Thus, it is important to develop novel processing methods to get good quality for soy proteins and soy products.

Pulsed electric field (PEF) is one of the new non-thermal processing methods for liquid foods, such as milk, juices, and liquid egg products. Successful applications of pasteurization with PEF technology for liquid products suggested that this technology can be a substitute for traditional thermal pasteurization or as a complement. Many studies of PEF have focused on the inactivation of microorganisms and enzymes by PEF treatments (Pothakamury et al., 1995; Ho et al., 1997; Martín et al., 1997; Reina et al., 1998; Wouters et al., 1999; Dutreux et al., 2000). The efficiency of PEF treatments in inactivation of microorganisms and enzymes depends on factors such as electric field intensity, pulse width, number of pulses, treatment time, and pulse frequency, among which electric field intensity and number of pulses are two critical factors (Barsotti et al., 2002; Bendicho et al, 2002; Jeyamkondan et al., 1999; Loey et al., 2002; Vega-Mercado et al., 1997; Wouters et al., 2001).

There are few reports on the effects of PEF on the structural modification of some enzymes (Yang et al., 2004; Zhong et al., 2005a; Zhong et al., 2005b; Zhang et al., 2007) and food proteins (Jeantet et al., 1999; Fernández-Díaz et al., 2000; Perez & Pilosof, 2004). No information is available regarding the effects of PEF on the structural modification of soy protein. The objective of this study was to investigate the effect of PEF on structural modification of soy protein. The intrinsic fluorescence intensity,

extrinsic fluorescence intensity, and surface hydrophobicity of SPI were monitored by fluorescence spectroscopy.

5.3 MATERIALS AND METHODS

5.3.1 Materials

BSA® soy protein isolate (SPI) powder (lot no. 2A2E003) was used in this study and provided by BSA Food Ingredients Inc. (St-Léonard, QC, Canada). The SPI powder product contains 90.0% soy protein (dry basis) measured by the supplier using the standard method. The extrinsic fluorescence spectroscopy probe, 1-anilino-8-naphthalenesulfonate (ANS) was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). All chemicals (such as sodium hydroxide or NaOH) used in this study were analytical grade, purchased from Fisher Chemicals (Fairlawn, NJ).

5.3.2 Preparation of samples

SPI powder was diluted to SPI solution (3%, w/v) with distilled water and stirred for 1 hr. The pH of the SPI solutions was adjusted to 7.0 with 0.1 M NaOH. Each SPI solution was mixed for additional 2 hr to ensure homogeneity. Afterwards, PEF treatments were applied to aliquots of the SPI solutions.

5.3.3 Experimental design

A two factor (electric field intensity and number of pulses) and two and four level factorial design was used to evaluate the effect of electric field intensities of 22 and 25 kV cm^{-1} and number of pulses of 30, 60, 90 and 120 on the structural modification and surface hydrophobicity of soy proteins.

5.3.4 Pulsed electric field (PEF) treatment

PEF treatments were performed using a high voltage decay generator TG2 and a batch parallel plate treatment chamber (TC-01001) as described in Chapter 3. The generator produced exponential decay pulses and pulse frequency was 0.5 Hz. The capacitance of the discharge capacitor of the generator was 0.33 μF. The batch parallel plate treatment chamber (TC-01001) was equipped with cooling jackets connected to the water bath set at 10°C in order to maintain the sample temperature not higher than 35°C. SPI sample temperature was measured within 20 sec before and after each PEF treatment, using a K type thermocouple (OMEGATM, Stamford, CT).

5.3.5 Intrinsic fluorescence spectroscopy

Intrinsic fluorescence of SPI was obtained by using an excitation wavelength of 290 nm and the emission spectra with the emission wavelength ranging from 305 to 400 nm, as described by Kulmyrzaev & Defour (2002); Stapelfeld & Skibsted (1999) and Garimella-Purna et al. (2005). The total absorbance of solutions with low protein concentration is typically constant, and the fluorescence intensity is proportional to the fluorophore concentration (Genot et al., 1992; Zhang et al., 2005). Therefore SPI solution used in this study was diluted with distilled water (pH 7.0) to yield a final protein concentration of 0.03% before its fluorescence intensity was measured. The intrinsic tryptophan fluorescence intensity of WPI was measured at room temperature (25 ± 1^{o}C) using a Hitachi F-2000 fluorospectrophotometer (Hitachi, Ltd., Tokyo, Japan) with a slit for excitation of 2.0 nm and a scanning speed of 60 nm min^{-1}.

5.3.6 Extrinsic fluorescence spectroscopy

The extrinsic fluorescence intensity was determined by using ANS as the extrinsic fluorescence probe (Sklar et al., 1977; Hayakawa & Nakai, 1985; Bonomi & Iametti, 1991). For the ANS fluorescence intensity measurement of SPI, 0.1 mL of ANS solution (10.0 mM in 0.05 M phosphate buffer, pH 7.0) was added to 4.9 mL of diluted SPI

solutions (0.03%, w/v). Then the mixed sample was left at room temperature (25±1^{o}C) for 1 hr to ensure homogeneity before the fluorescence measurements were performed. Extrinsic ANS fluorescence intensity of SPI was assayed by using an excitation wavelength of 375 nm and the emission wavelength ranging from 420-560 nm using a Hitachi F-2000 fluorospectrophotometer as described by Boatright & Hettiarachchy (1995).

5.3.7 Surface hydrophobicity of SPI using ANS probe

Surface hydrophobicity of SPI solutions (3%, w/v) was measured according to Kato & Nakai (1980) using fluorescence probe ANS. PEF-treated and non-treated (control) SPI solutions were diluted with distilled water to a final concentration of 0.03%. Then, 0.1 mL of ANS (50 mM in 0.05 M phosphate buffer, pH =7.0) was added to 4.9 mL of the diluted SPI sample and mixed. Extrinsic fluorescence intensity of SPI solutions was measured at room temperature (25 ± 1^{o}C) with a Hitachi F-2000 fluorospectrophotometer at excitation wavelength of 375 nm and the emission wavelength ranging from 420-560 nm. Surface hydrophobicity in SPI solutions was expressed as the ratio of the extrinsic fluorescence intensity and protein concentration of SPI as described by Li et al. (2007).

5.4 STATISTICAL ANALYSIS

All experiments were conducted with three replicates; means and standard deviations were reported. Analysis of variance (ANOVA) was undertaken with SAS software (SAS 9.1, SAS Institute Inc., Cary, NC, USA) for all data analysis. Main effects were considered significant when $P \leq 0.05$ level.

5.5 RESULTS AND DISCUSSION

5.5.1 Intrinsic tryptophan fluorescence intensity

PEF treatments of the electric field intensity (22 and 25 kV cm^{-1}) and the number of pulses (30, 60, 90 and 120) affected the intrinsic fluorescence intensity of SPI solution as shown in Table 5.1. All PEF treatments resulted in increases in the intrinsic fluorescence intensity of SPI solutions and all obtained values were significantly ($P \leq 0.05$) greater than that of the control (13.65 ± 0.15 a.u.). Increases in the intrinsic fluorescence intensity of PEF-treated SPI solutions were significantly different ($P \leq 0.05$) as shown in Table 5.1

Table 5. 1 Electric field intensity and number of pulses effects on the intrinsic fluorescence intensity of SPI (at the emission wavelength of 337 nm).

Electric field intensity (kV cm^{-1})	Number of pulses (n)			
	30	60	90	120
22	B 14.21^{d}	B 14.60^{c}	B 15.07^{b}	B 15.61^{a}*
25	A 14.43^{d}	A 15.02 ^{c}	A 15.63^{b}	A 16.35^{a}

*****Values with same letters in the row (a, b, c and d) are not significantly different ($P > 0.05$) and means with same letters in the column (A and B) are not significantly different ($P > 0.05$), standard deviation for each value was not shown here.

The intrinsic fluorescence emission spectra of SPI treated at 22 kV cm^{-1} are shown in Figure 5.1. Intrinsic fluorescence intensities of SPI solutions increased with increasing number of pulses from 13.65 a.u. (the control) to 14.21, 14.60, 15.07 and 15.61 a.u. for 30, 60, 90 and 120 pulses, respectively. The emission spectrum for the control showed the maximum fluorescence intensity at the emission wavelength of 337 nm. As the degree of structural modification increased with PEF treatments, a progressive increase in the red shift was observed with a maximum shift of 6 nm. PEF treatment of 22

kV cm^{-1} and 90 pulses resulted in a red shift of 2 nm (337 to 339 nm), while PEF treatment of 22 kV cm^{-1} and 120 pulses resulted in a red shift of 4 nm (337 to 341 nm). This results indicated that PEF treatments (increasing number of pulses) caused some expansion and fragmentation of soy protein molecule resulting in the exposure of more tryptophan residues to the polar environment and thus to a higher red shifts. Achouri & Zhang (2001) also obtained red shifts in the tryptophan fluorescence emission spectra of soy protein with succinylation.

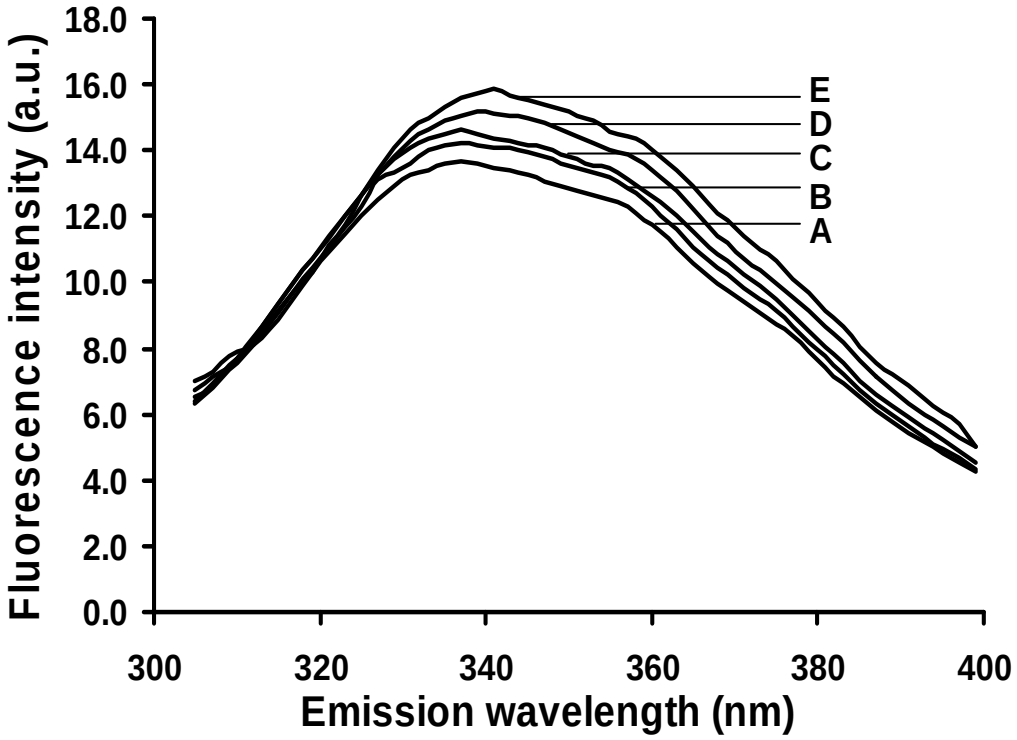

Figure 5. 1 Intrinsic tryptophan fluorescence emission spectra of the control (no PEF treated) and PEF-treated (22 kV cm^{-1}) SPI samples. The number of pulses was: (A), control; (B), 30 pulses; (C), 60 pulses; (D), 90 pulses and (E), 120 pulses.

The intrinsic fluorescence emission spectra of SPI treated with PEF treatments at 90 pulses are shown in Figure 5.2. Red shifts of the emission wavelength were observed

with PEF treatments. PEF treatment of 22 kV cm^{-1} and 90 pulses resulted in a red shift of 2 nm (337 to 339 nm) in the emission wavelength, while PEF treatment of 25 kV cm^{-1} and 90 pulses resulted in a red shift of 4 nm (337 to 341 nm). Intrinsic fluorescence intensities of SPI significantly increased with increasing electric field intensity, e.g., 15.07 and 15.63 a.u. for 22 and 25 kV cm^{-1}, respectively. Increase in the intrinsic fluorescence intensity and the red shifts indicated changes in the polarity of tryptophan residues microenvironment of soy proteins from a less polar to a more polar environment. The results confirmed the findings of Moro et al. (2001) that protein unfolding caused a red shift on the fluorescence emission spectrum due to exposure of the tryptophan residues to the aqueous solvent. It could be concluded that tryptophan residues of modified soy proteins may be in a more polar environment than those of native proteins when the proteins were in the polar solvent (Kim & Rheei, 1989).

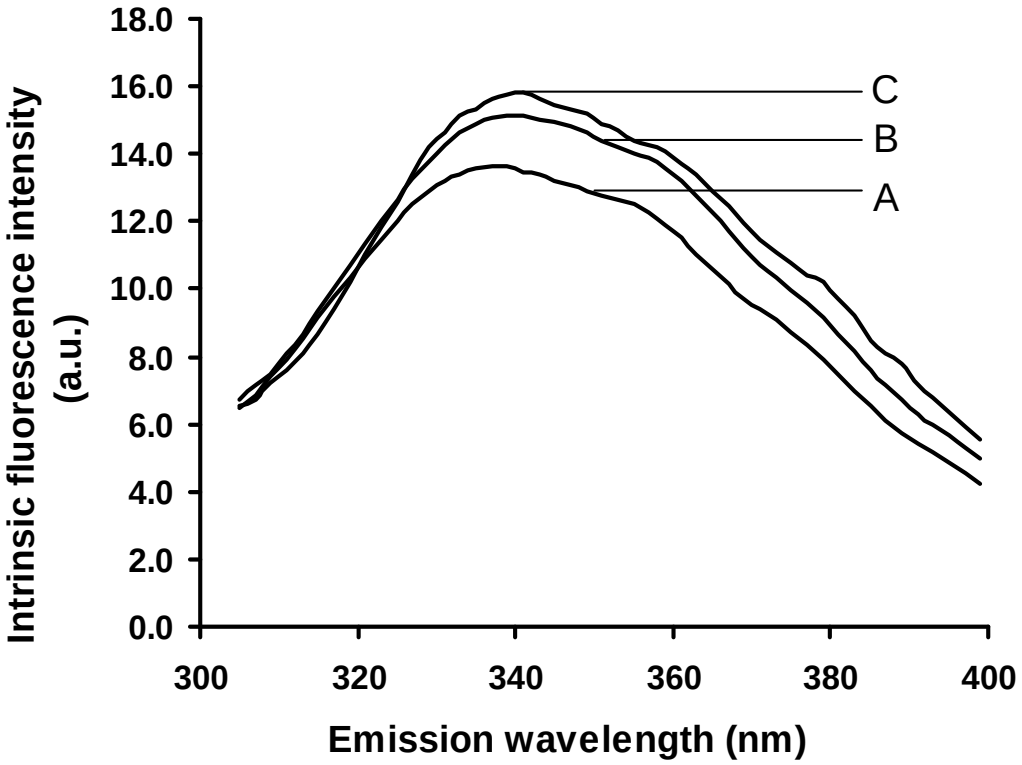

Figure 5. 2 Intrinsic tryptophan fluorescence emission spectra of the control (no PEF treated) and PEF-treated (90 pulses) SPI samples. The electric field intensities were: (A), control; (B), 22 kV cm^{-1}; (C), 25 kV cm^{-1}.

5.5.2 Extrinsic fluorescence intensity

PEF treatments of the electric field intensity (22 and 25 kV cm^{-1}) and the number of pulses (30, 60, 90 and 120) affected the extrinsic fluorescence intensity of SPI solution as shown in Table 5.2. All PEF treatments (different electric field intensities and different pulses) significantly ($P \leq 0.05$) increased the extrinsic fluorescence intensity of SPI solutions over the control (12.92 ± 0.11 a.u.). The increase in extrinsic fluorescence intensities of SPI for treatments using 30, 60, 90 and 120 pulses were 0.78, 1.37, 1.98 and 2.70 a.u., respectively at the electric field intensity of 25 kV cm^{-1} whereas when the electric field intensity of 22 kV cm^{-1} was used, the extrinsic fluorescence intensities were 0.56, 0.95, 1.42 and 1.96 a.u., respectively. These results confirmed that soy protein

fractions were partially denatured with PEF treatments. The effect of PEF increased with increasing intensity of treatment. The results were similar to those observed for soy protein in soy milk under high pressure treatments (Zhang et al., 2005). The increase in the extrinsic fluorescence intensity could be due to the exposure of higher number of hydrophobic groups binding to the ANS-protein due to intermolecular interactions (Hayakawa et al., 1992; Tanaka et al., 1996; Zhang et al., 2005).

Table 5. 2 Electric field intensity and number of pulses effects on the extrinsic fluorescence intensity of SPI (at the emission wavelength of 482 nm).

Electric field intensity ($kV\ cm^{-1}$)	Number of pulses (n)			
	30	60	90	120
22	B13.26d*	B13.56c	B14.02b	B14.607a
25	A13.49d	A13.93c	A14.58b	A15.267a

*****Values with same letters in the row (a, b, c and d) are not significantly different ($P > 0.05$) and means with same letters in the column (A and B) are not significantly different ($P > 0.05$), standard deviation for each value was not shown here.

The extrinsic fluorescence spectra of SPI with PEF treatments at 22 $kV\ cm^{-1}$ and different pulses are presented in Figure 5.3. The extrinsic fluorescence intensities of SPI increased significantly ($P \leq 0.05$) with increasing number of pulses. The maximum extrinsic fluorescence intensity was obtained at the emission wavelength of 482 nm for the control. Blue shifts of 2-4 nm occurred in the emission wavelength of the maximum fluorescence intensity of SPI emission spectra following PEF treatments of 22 $kV\ cm^{-1}$ and different pulses (30, 60, 90 and 120). These blue shifts indicated that the PEF-treated SPI bound more ANS in the more polar environment compared to the native SPI. Similar results were obtained by Zhang et al. (2005) for soy protein denaturation after high pressure treatments of 400 MPa.

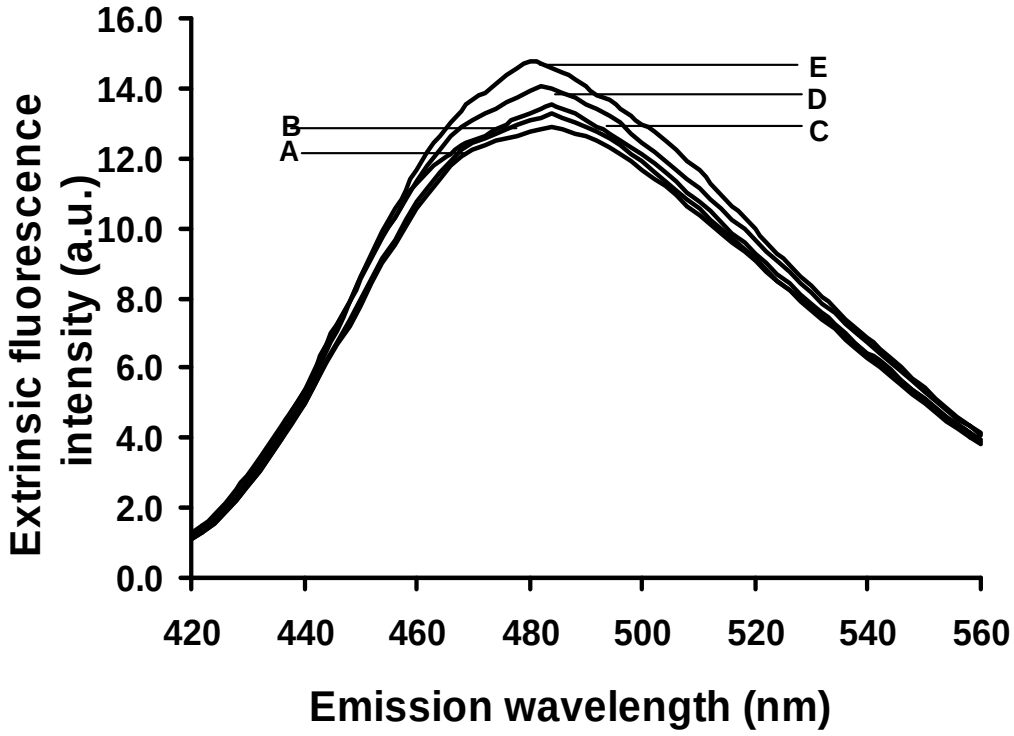

Figure 5. 3 Extrinsic fluorescence emission spectra of the control (no PEF treated) and PEF-treated (22 kV cm^{-1}) SPI samples. The number of pulses was: (A), control; (B), 30 pulses; (C), 60 pulses; (D), 90 pulses and (E), 120 pulses.

5.5.3 Changes of surface hydrophobicity with PEF treatments

Changes of surface hydrophobicity of SPI solutions with PEF treatments of 25 kV cm^{-1} and different pulses are shown in Figure 5.4. Surface hydrophobicity of SPI was significantly greater ($P \leq 0.05$) after PEF treatments than before. Surface hydrophobicity of SPI increased with increasing number of pulses, reaching the maximum value (108% of the control) at 25 kV cm^{-1} and 60 pulses. Surface hydrophobicity reached the maximum value (118% of the control) at 25 kV cm^{-1} and 120 pulses. The results were similar to the results of Li et al. (2007) who reported that SPI exposed at 30 kV cm^{-1} and treatment time of 288 μs showed 120% of the control. Molina et al. (2001) showed that an increase in surface hydrophobicity of soy proteins occurred, presumably due to partial

denaturation, and subsequent exposure of hydrophobic groups with 200 MPa high-pressure treatments at neutral pH. Puppo et al. (2004) obtained similar results and they reported that high pressures higher than 200 MPa increased surface hydrophobicity, partially unfolding soy protein fractions and inducing the aggregation and dissociation of SPI proteins. Other PEF treatments (electric field intensity of 20-35 kV cm^{-1}, 100-900 Hz frequency, 2-8 pulses) did not induce denaturation of diaultrafiltered egg white protein by measuring surface hydrophobicity, probably due to different PEF systems (Jeantet et al., 1999).

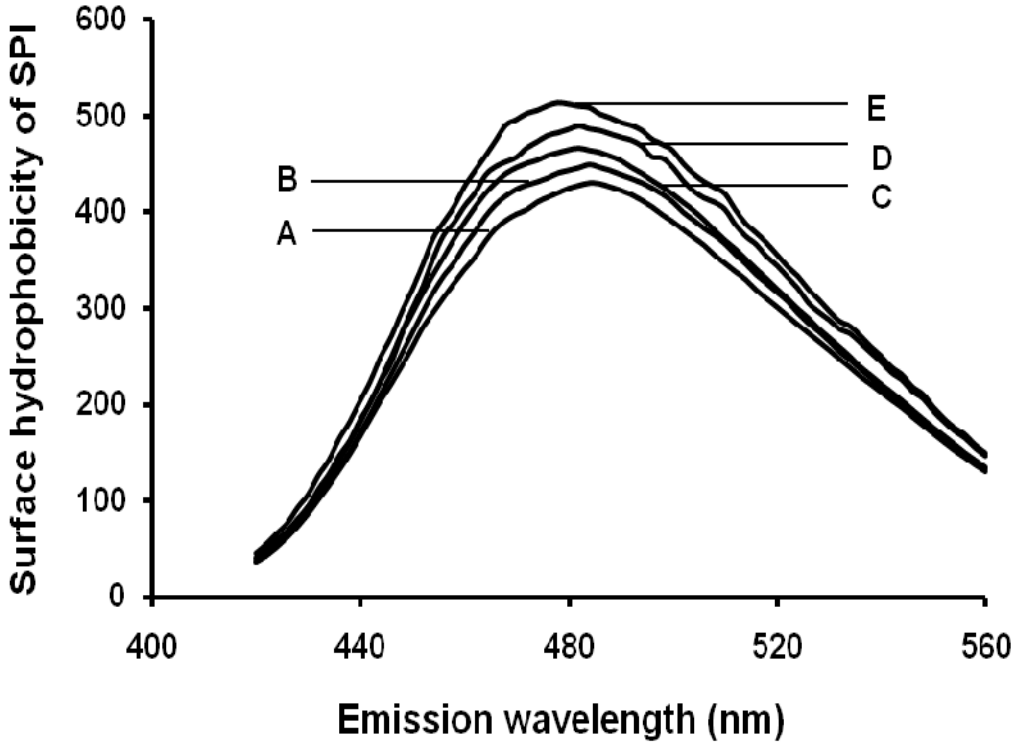

Figure 5. 4 Surface hydrophobicity changes of the control (no PEF treated) and PEF-treated (25 kV cm^{-1}) SPI samples. The number of pulses was: (A), control; (B), 30 pulses; (C), 60 pulses; (D), 90 pulses and (E), 120 pulses.

5.6 CONCLUSION

The effects of PEF on the structural modification and surface hydrophobicity of SPI were studied. The results of this study indicated that electric field intensity and number of pulses both partially modified protein structure of SPI. The intrinsic tryptophan fluorescence intensity of SPI increased and red shifts of 2-6 nm occurred after PEF treatments. Extrinsic fluorescence intensity changes and blue shifts of SPI were related to the structural modification of SPI with the PEF treatments. The surface hydrophibicity of SPI increased with increasing electric field intensity or number of pulses, confirming that these were main parameters influencing structural modification of SPI and their effects were closely related to their effects on the structural modification of SPI.

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CONNECTING TEXT

A comprehensive review of literature demonstrated the need for a study regarding the effects of pulsed electric fields (PEF) on the physicochemical properties of food proteins especially rheology. The following study sought to determine the changes of rheological properties of reconstituted skim milk.

Chapter 6 describes how the rheological properties of reconstituted skim milk were affected by PEF treatments and evaluated using a rheometer. PEF treatments were used with different electric field intensities of 15, 18 and 20 kV cm^{-1} and number of pulses of 20, 40 and 60. The results of this study confirmed that the changes of flow behavior and rheological properties of reconstituted skim milk took place under these PEF conditions. The results confirmed that PEF treatments changed the rheological properties of reconstituted skim milk, and that PEF treatments could change the functional properties of proteins due to changes of rheological properties of food proteins.

6. FLOW BEHAVIOR AND VISCOSITY OF RECONSTITUTED SKIM MILK TREATED BY PULSED ELECTRIC FIELDS

6.1 ABSTRACT

Flow behavior and viscosity are the most important functionalities of food proteins. They are very important for providing physical stability to emulsions and other suspended particles in foods and contributing to the mouthfeel and good quality of foods. This study dealt with the effects of pulsed electric fields (PEF) on flow behavior and viscosity of reconstituted skim milk. The changes in flow behavior and viscosity were monitored for each skim milk sample. Steady-state and peak hold tests of reconstituted skim milk with different PEF treatments were investigated. The flow behavior and viscosity of PEF-treated reconstituted skim milk were studied using a controlled stress rheometer. The reconstituted skim milk was subjected to 20, 40 or 60 pulses of electric field intensity of 15, 18 and 20 kV cm^{-1} with a system capacitance of 0.33 μF. Rheological properties were then measured, with shear rate from 0 to 200 s^{-1} and 200 to 0 s^{-1}. Reconstituted skim milk showed shear-thinning behavior (flow behavior index n = 0.96-0.99). The shear rate and the shear stress data of PEF-treated reconstituted skim milk were described with the Herschel-Bulkley model. The apparent viscosity of reconstituted skim milk increased with increasing electric field intensity and number of pulses. It increased from 2.12 to 4.25 (10^{-3} Pa.s) for the control and the PEF condition of 60 pulses and 20 kV cm^{-1}. The consistency index (K) also increased with increasing electric field intensity and number of pulses. The consistency index (K) increased from 1.42 to 1.54 (10^{-3} $Pa.s^n$) for the control and the PEF-treated at 60 pulses and 20 kV cm^{-1}. The changes of rheological parameters of reconstituted skim milk with PEF treatments were confirmed by rheology measurements. The results confirmed that PEF treatments affected rheological properties of food proteins.

Keywords: Reconstituted skim milk, pulsed electric field (PEF), rheological property, flow behavior, flow model, viscosity.

6.2 INTRODUCTION

Milk is a very popular product in daily life. Milk contains milk proteins, fat globules, carbohydrates, minerals, and water. It is well established that milk composition and processing treatments affect the microstructure of protein coagulum. Milk treatments performed in dairy plants, such as ultrafiltration, homogenization and heating, modify the fat particle size and change the milk fat globule membrane and protein denaturation (Lopez & Dufour, 2001). Heat treatments induce milk protein aggregation because of protein denaturation and subsequent protein-protein interaction via calcium ion and disulfide bond formation during sterilization of milk (Schene & Morr, 1996).

Flow behavior and viscosity are the most important functional properties of food proteins. They are very important for providing physical stability to emulsions and other suspended particles in foods and contribute to the mouth-feel of foods (Schene & Morr, 1996). Processing-induced changes in proteins such as polymerization, aggregation and hydrolysis affect the flow behavior and viscosity of food products. The concentrations and inherent physicochemical properties, i.e. molecular weight, polydispersity, hydrophobicity, and conformation of each protein aspects, affect the flow behavior and viscosity of the protein solution. The physicochemical properties of other ionic and nonionic solutes also influence the flow behavior and viscosity of protein solutions by contributing directly to viscosity and also by their tendency to interact and modify the physicochemical properties of the proteins. All of these factors tend to confound the underlying inverse relationship of protein solubility and viscosity (Shen, 1981). Protein concentration, dispersion conditions, pH and ionic strength can affect protein solubility and viscosity of protein solutions. The viscosity of proteins is both high sensitive to the history of the protein and the techniques used to measure it (Shen, 1981). Other factors which affect the viscosity of proteins include processing and compositional factors, temperature, pH of precipitation, calcium content and high pressure.

PEF technology is of specific interest for the food industry and it not only provides attractive alternatives to conventional thermal processing methods which often produce undesirable changes in food hampering the balance between high quality and safety (i.e. color, flavor, functionality), but also it offers opportunities for creating new ingredients and products due to the specific actions on biological materials and food constituents. PEF allows redesigning of existing processes and creating entirely new ones alone or in combination with conventional processes (e.g. PEF-temperature combination). As a result, some researchers have investigated the use of PEF treatments on milk, their effects on the physicochemical characteristics of milk and in terms of quality improvement. For example, Perez & Pilosof (2004) found that PEF induced structural changes in proteins and modifies their functional performance. β-Lactoglobulin (β-LG) (10%, w/v, pH 7) was subjected to PEF treatments of 1 to 10 pulses and the electric field intensity of 12.5 kV cm^{-1}. β-Lactoglobulin was increasingly denatured by increasing the number of pulses. Bendicho et al. (2002b) evaluated PEF effects on several water-soluble vitamins and fat-soluble vitamins in milk. Milk retained more ascorbic acid after PEF treatment of 22.6 kV cm^{-1} and treatment time of 400 μs than those after either a low or high heat-pasteurization treatment.

The effects of PEF on milk have been studied to evaluate the level of microbial and enzyme inactivation. This has been found to mainly depend on electric field intensity and number of pulses during PEF processes (Vega-Mercado et al., 1996; Sensoy et al., 1997; Reina et al., 1998; Grahl & Märkl, 1996; Ho et al., 1997; Barsotti et al., 2002; Martín et al., 1997; Bendicho et al., 2002a). However, no studies have investigated that PEF treatments effect on the flow behavior and viscosity of milk or milk products. Therefore, it is very interesting to study flow behavior and viscosity of milk as a function of PEF treatments because flow behavior and viscosity are the most important functional properties of food proteins in milk processing.

In this study, the effects of PEF treatments on the flow behavior, rheological parameters and viscosity of reconstituted skim milk were studied. The specific objective was to study flow behavior, flow model and viscosity of skim milk after it had been

exposed to electric field intensities of 15, 18 and 20 kV cm^{-1} and number of pulses of 20, 40 and 60.

6.3 MATERIALS AND METHODS

6.3.1 Sample preparation

Low-heat skim milk powder manufactured by Loblaw's Inc., Toronto, Canada, was purchased from a local food market. This milk powder contained ~36% protein on a dry basis. Experimental skim milk samples were prepared by skim milk powder to 5% total solids (w/v) in distilled water. The reconstituted skim milk samples were allowed to equilibrate at ambient temperature (~20°C) with gentle stirring for at least 4 h before further PEF treatments. A small amount of sodium azide (0.01%, w/v) was added to all milk samples as a preservative.

6.3.2 Pulsed electric field treatments

PEF treatments were carried out by using a high voltage pulse generator TG2 (Food Process Engineering Laboratory, Macdonald Campus of McGill University). The simplified electric circuit for pulse generator is shown in Figure 6.1. It consisted of a variable autotransformer (Powerstat Type 3PN116C) which supplied voltage to the circuit. The voltage was elevated by high voltage transformer (62159A - Apotex Inc., Weston, ON, Canada) and then rectified by a high voltage diode. The voltage across the treatment chamber depended on the distance between the spheres of the stainless steel, 15 mm diameter discharger. The break voltage for this diameter was calculated by the following equation (Armyanov et al., 2001).

$$V_o = 4.85h^{0.75} \quad (1)$$

where h is the distance between the spheres of the discharger (cm), V_o is the break voltage (kV).

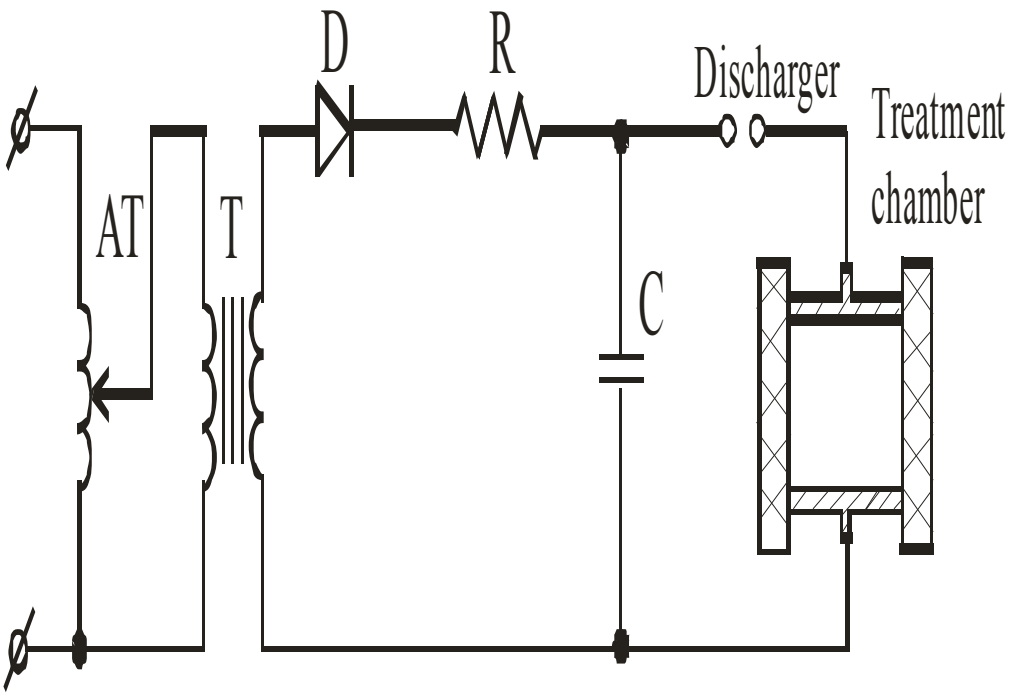

Figure 6. 1 Simplified electric circuit diagram for the high voltage pulse generator used to treat reconstituted skim milk

The treatment chamber (TC-01004) had an active area of 3.8 cm^2, a volume of 4.6 mL and a gap between the two electrodes of 1.0 cm. The skim milk sample was PEF-treated with electric field intensities of 15, 18 and 20 kV cm^{-1}, capacitance of discharge capacitor of 0.33 μF and number of pulses (20, 40 and 60) in series of 20 pulses at the pulse frequency of 0.50 Hz. Intervals of 3 min were allowed between 20 pulses series to permit heat removal from the treatment chamber through the steel electrode. The sample temperature of the treatment chamber was measured within 30 sec before and after each PEF treatment using a *K* type thermocouple (OMEGATM, Stamford, CT). The sample temperature was not higher than 35^oC with each PEF treatment. The voltage and current across the treatment chamber were monitored using a two channel digital oscilloscope (54621A, Agilent Technologies, Inc., CA). The PEF-treated samples were immediately transferred to refrigerator at 4^oC and the flow behavior and viscosity of skim milk sample were studied within 12 hr.

6.3.3 Flow behavior and viscosity measurements

Flow behavior and viscosity measurement of reconstituted skim milk sample were carried out using a controlled stress rheometer (AR 2000, TA Instruments, New Castle, DE, USA). A double concentric cylinder was used to measure the flow behavior and viscosity of reconstituted skim milk sample. Each test of skim milk sample of 6.48 mL was transferred to the sample compartment. The temperature was set at 20^{o}C throughout the entire measurement. The flow behavior of reconstituted skim milk was evaluated by two-cycle shear rate changes from 0 to 200 s^{-1} for combination in 10 min and then from 200 to 0 s^{-1} in next 10 min. Three replicates were conducted for all flow behavior measurements. Viscosities of skim milk with different PEF treatments were measured by using a controlled stress rheometer at a shear rate of 10 s^{-1}.

The TA instrument's (Rheology Advantage, TA version 5.1) software was used for conducting data analysis. The rheological parameters were estimated by model fitting using the data analysis to minimize the standard error (Kristensen et al., 1996). The choice of a flow model was based on the evaluation of standard error and regression values which fit to the models. The model that best fits at any PEF-treated conditions was selected.

6.4 STATISTICAL ANALYSIS

All experiments were conducted with three replicates; means and standard deviation were reported. Analysis of Variance (ANOVA) was undertaken with SAS software (SAS 9.1, SAS Institute Inc., Cary, NC, USA) for all data analysis.

6.5 RESULTS AND DISCUSSION

6.5.1 Flow models

Different rheological models (Newtonian, Bingham, Casson, Power law and Herschel Bulkley) were evaluated to describe shear stress and shear rate data of the PEF-treated skim milk. Flow curves (shear stress compared to shear rate) were determined at

increasing and decreasing shear rates between 0 and 200 s^{-1} in 10 min (up and down flow curves). The Herschel-Bulkley model shown as below described the flow behavior more adequately.

$$\tau = \tau_o + K(\gamma)^n, \quad (2)$$

Where, τ is shear stress (Pa); τ_o is yield stress (Pa), γ is shear rate (s^{-1}); K is consistency index ($Pa.s^n$); n is flow behavior index (dimensionless). It was observed that the Herschel-Bulkley model fitted adequately (Table 6.1) when skim milk was treated by PEF treatment at electric field intensity of 20 kV cm^{-1} and 20 pulses.

Table 6. 1 Fitting of flow models for PEF-treated skim milk at an electric field intensity of 20 kV cm^{-1} and 20 pulses.

Model	Yield stress, τ_o (10^{-3} Pa)	Consistency index (K) (10^{-3} $Pa.s^n$)	Flow behavior index (n)	Standard error
Newtonian	/	1.80	/	51.62
Bingham	53.2	1.34	/	5.25
Casson	23.5	1.06	/	6.43
Power law	/	5.08	0.773	9.23
Herschel-Bulkley	39.6	1.46	0.988	3.28

6.5.2 Effect of PEF on the flow behavior of reconstituted skim milk

Shear stress was plotted against shear rate to examine the flow behavior of PEF-treated skim milk samples. Figure 6.2 shows the shear stress versus shear rate plot of reconstituted skim milk with PEF treatments. The profiles followed predictable trends with the electric field intensity: the shear stress increased with increasing electric field intensity. The flow behavior for electric field intensity of 15, 18 and 20 kV cm^{-1} was the

Herschel-Bulkley model. The result confirmed that the electric field intensity had a strong effect on the shear stress of the reconstituted skim milk.

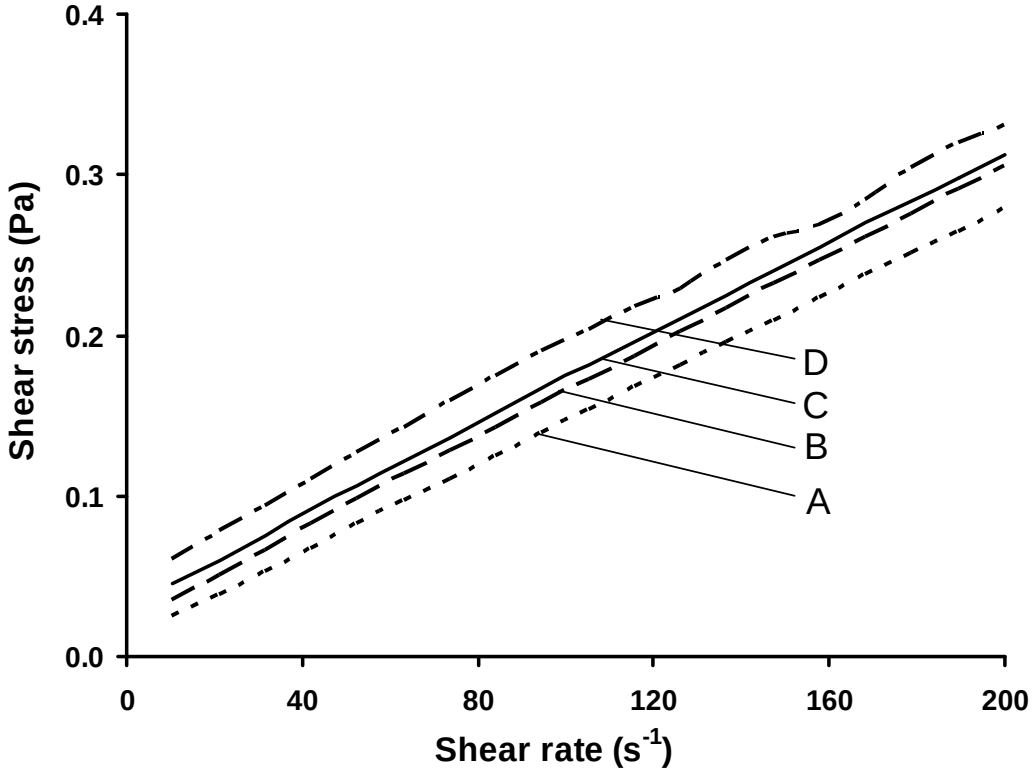

Figure 6. 2 Shear stress versus shear rate plot of reconstituted skim milk applied to PEF treatments. (A), Control; (B), 15 kV cm^{-1}; (C), 18 kV cm^{-1} and (D), 20 kV cm^{-1}.

The electric field intensity (15, 18 and 20 kV cm^{-1}) and number of pulses (20, 40 and 60) affected the flow behaviour of skim milk as shown in Table 6.3. All PEF treatments resulted in decreases in the flow behaviour (n = 0.9920 ~ 0.9603) of skim milk and all obtained values were not significantly ($P > 0.05$) smaller than that of the control (0.994). The results indicated that electric field intensity and number of pulses both decreased the flow behaviour. Comparison of means indicated that there was no significant difference between *n* values for the control and the PEF-treated skim milk samples. However, increase in electric field intensity from 15 to 20 kV cm^{-1} lowered *n*

values and increased shear-thinning behavior of reconstituted skim milk. The degree of shear thinning pseudoplasticity typically increased with increasing electric field intensity in reconstituted skim milk because the flow behavior index (*n*) decreased from 0.99 to 0.96 as shown in Table 6.3.

Table 6. 2 The electric field intensity and number of pulses effects on flow behavior of skim milk.

Electric field intensity (kV cm^{-1})	Number of pulses (n)		
	20	40	60
15	A 0.9903[a*]	A 0.9803[b]	A 0.9706[c]
18	A 0.9920[a]	A 0.9796[b]	A 0.9693[c]
20	A 0.9906[a]	B 0.9703[b]	B 0.9603[c]

*****Values with same letters in the row (a, b and c) are not significantly different ($P > 0.05$) and means with same letters in the column (A and B) are not significantly different ($P > 0.05$), standard deviation for each value was not shown here.

6.5.3 Effect of PEF on apparent viscosity of reconstituted skim milk

The electric field intensity (15, 18 and 20 kV cm^{-1}) and number of pulses (20, 40 and 60) affected the apparent viscosity of skim milk as shown in Table 6.3. All PEF treatments resulted in increases in the apparent viscosity of skim milk and all obtained values were significantly ($P \leq 0.05$) greater than that of the control (2.12 ± 0.32 (10^{-3} Pa.s)). The results indicated that electric field intensity and number of pulses both increased the apparent viscosity of skim milk.

Table 6. 3 The electric field intensity and number of pulses effects on apparent viscosity of skim milk.

Electric field intensity ($kV\ cm^{-1}$)	Number of pulses (n)		
	20	40	60
15	C 2.620^{c*}	C 3.260^{b}	C 3.610^{a}
18	B 2.653^{c}	B 3.423^{b}	B 4.063^{a}
20	A 2.723^{c}	A 3.546^{b}	A 4.256^{a}

*****Values with same letters in the row (a, b and c) are not significantly different ($P > 0.05$) and means with same letters in the column (A, B and C) are not significantly different ($P > 0.05$), standard deviation for each value was not shown here.

Typical results of the apparent viscosity determination for reconstituted skim milk with PEF treatments are shown in Figure 6.3, showing the apparent viscosity of reconstituted skim milk as a function of shear rate. As the number of pulses increased, a marked increase in the apparent viscosity was observed at all shear rates. Up to 40 pulses, the apparent viscosity seemed to be independent of shear rate. There is a visible change in flow characteristics above 40 pulses. For all skim milk samples, the apparent viscosity decreased rapidly with increasing shear rate until the shear rate reached 75 s^{-1} and then decreased slowly with shear rate until it decreased to the minimum value at the shear rate of 200 s^{-1}. The reconstituted skim milk can be characterized as shear-thinning fluids, with the flow curves lacking a linear characteristic. A rapid breakdown of the protein structure occurs on initial shearing followed by much slower changes at higher shear rates. This behavior can be attributed to combined effects of breakdown of weak linkages between the proteins and/or between the proteins and stabilizer, and/or reformation of such linkages as a result of Brownian motion and molecular collisions (Tang et al., 1993).

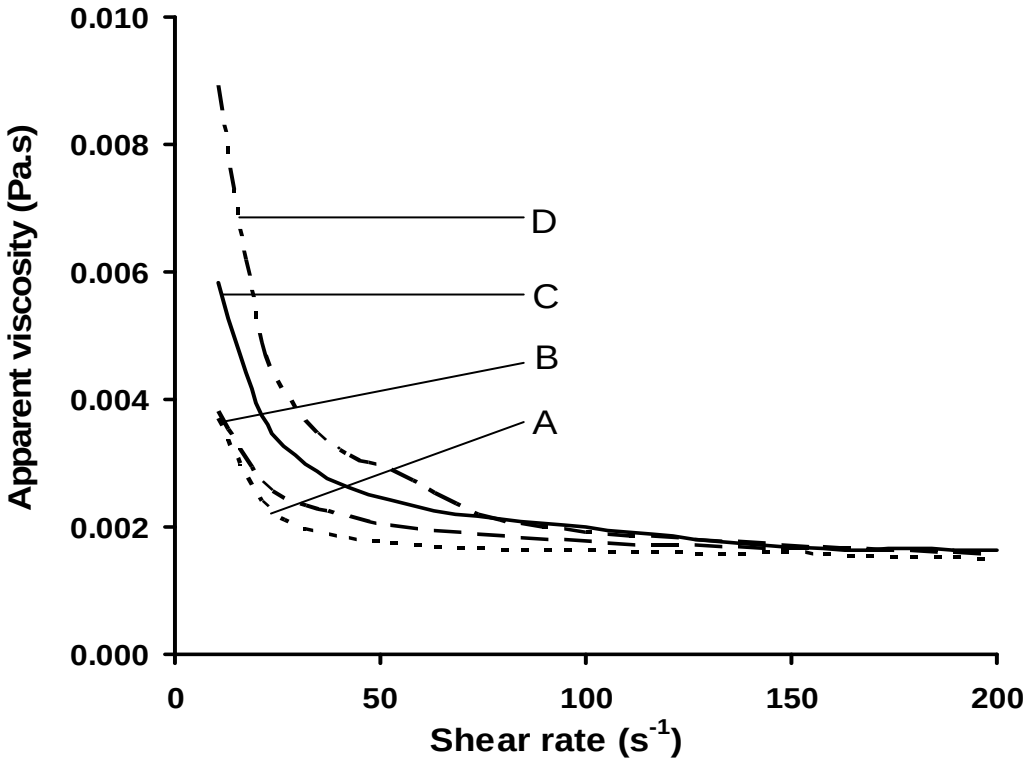

Figure 6. 3 Apparent viscosity of reconstituted skim milk as a function of shear rate at 20 kV cm^{-1}. (A), Control; (B), 20 pulses; (C), 40 pulses and (D), 60 pulses.

The comparison of apparent viscosity change for reconstituted skim milk with PEF treatments as a function of shear rate are shown in Figure 6.4. From this figure, the apparent viscosities of skim milk were seen to decrease with an increase in the shear rate from 0 to 200 s^{-1} when the electric field intensity increased from 15 to 20 kV cm^{-1}. But the apparent viscosity of skim milk was very different with and without PEF treatments. The apparent viscosity of the control decreased from 2.43 to 1.40 (10^{-3} Pa.s) with increasing shear rate from 0 to 200 s^{-1}. The apparent viscosity of the PEF-treated skim milk decreased from 5.82 to 1.62 (10^{-3} Pa.s) for an electric field intensity of 20 kV cm^{-1} with increasing shear rate from 0 to 200 s^{-1}. A possible reason for a decrease in viscosity with an increase in shear rate could be that whey protein coagulation and fat globule size changes in skim milk were initiated by PEF treatments.

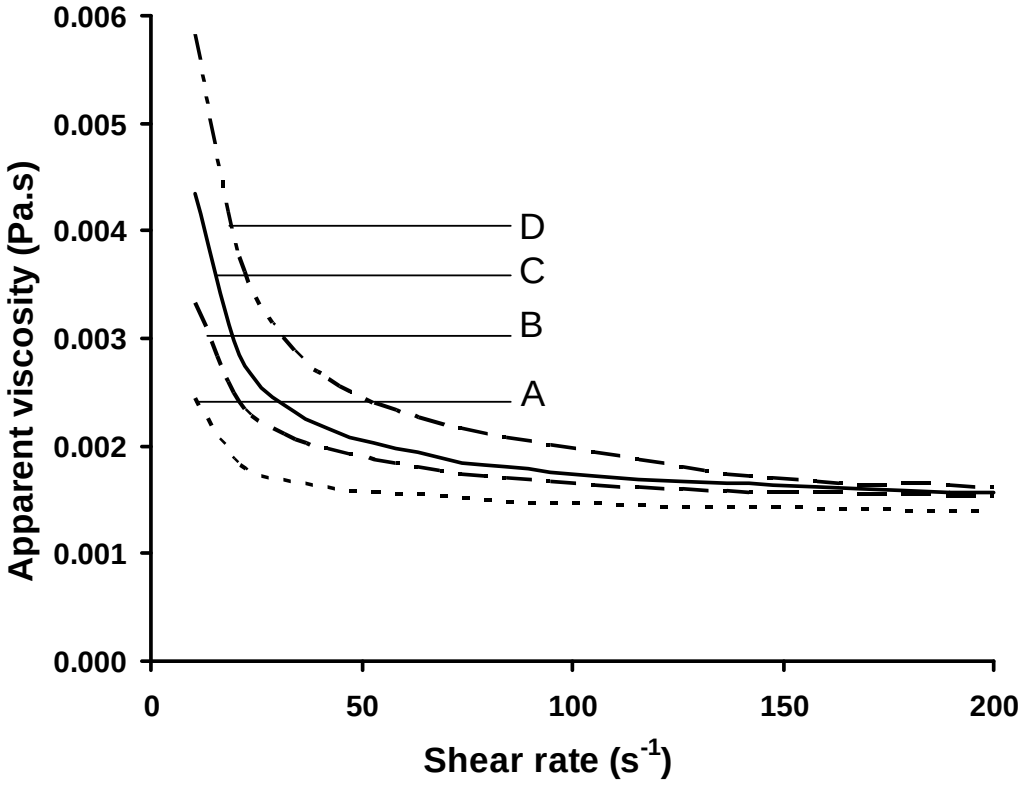

Figure 6. 4 Apparent viscosity of reconstituted skim milk as a function of shear rate with PEF treatments. (A), Control; (B); 15 kV cm^{-1}; (C), 18 kV cm^{-1} and (D), 20 kV cm^{-1}.

A completely different picture of apparent viscosity variation of reconstituted skim milk was observed after PEF treatments of the electric field intensity of 15, 18 and 20 kV cm^{-1} and number of pulses of 20, 40 and 60 (Figure 6.5). All PEF treatments (different electric field intensities and different number of pulses) increased the apparent viscosity of skim milk samples and all values were significantly ($P \leq 0.05$) greater than that of the control (2.12 ± 0.3 (10^{-3} Pa.s)). Increases in the apparent viscosity of PEF-treated skim milk samples were significantly different ($P \leq 0.05$). When electric field intensity increased to the maximum value of 20 kV cm^{-1} and 60 pulses, the apparent viscosity of reconstituted skim milk increased to the maximum value of 4.25 (10^{-3} Pa.s). The apparent viscosity showed a considerable increase with increasing electric field intensity and number of pulses. In reconstituted skim milk, an increase in the apparent

viscosity with PEF treatments was supposed to reflect intermolecular interactions resulting from the attractions between adjacent denatured molecules, with the formation of weak transient networks. These interactions would cause an increased effective volume. As the protein aggregation became larger, they occupied more space and thus contributed to an increase in the apparent viscosity of the fluid system (Adapa et al., 1997). Increases in the apparent viscosity of skim milk with PEF treatments were the same as the milk with heat treatments. Jeurnink & de Kruif (1993) confirmed that the apparent viscosity of milk was affected by heating. At temperatures above 70^{o}C, the viscosity of the milk increased during the early stages of heating and plateaued on prolonged heating. Bienvenue et al. (2003) also confirmed that the apparent viscosity of skim milk increased with heat treatments. The changes in milk viscosity were attributed to the association of denatured whey proteins with the casein micelles. These association reactions increased the size of the casein micelles and also influenced the interactions between the particles (Jeurnink & de Kruif, 1993).

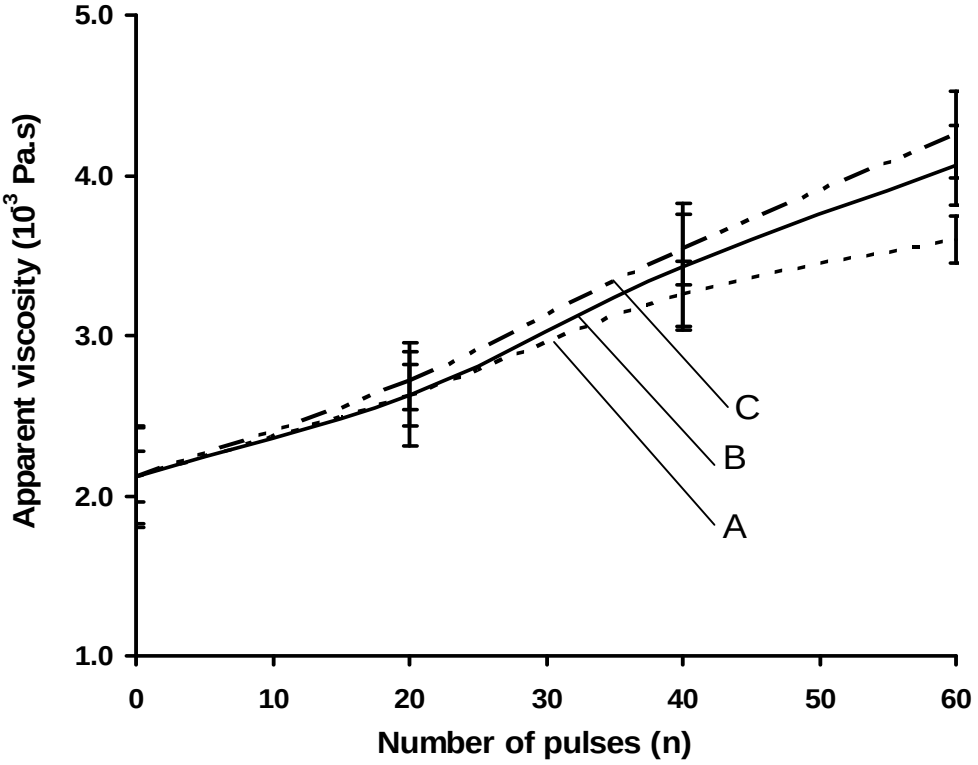

Figure 6. 5 Effect of electric field intensity and number of pulses on the apparent viscosity of skim milk. (A), 15 kV cm^{-1}; (B), 18 kV cm^{-1} and (C), 20 kV cm^{-1}.

6.5.4 Effect of PEF on consistency index of reconstituted skim milk

The electric field intensity (15, 18 and 20 kV cm^{-1}) and number of pulses (20, 40 and 60) affected the consistency index of skim milk as shown in Table 6.4. All PEF treatments resulted in increases in the consistency index of skim milk and all obtained values were significantly ($P \leq 0.05$) greater than that of the control (1.42 ± 0.01 (10^{-3} $Pa.s^n$)). The consistency index of skim milk after exposure to PEF at 15, 18, and 20 kV cm^{-1} ranged from 1.45 to 1.50 (10^{-3} $Pa.s^n$), 1.47 to 1.52 (10^{-3} $Pa.s^n$) and 1.50 to 1.55 (10^{-3} $Pa.s^n$) for the number of pulses of 20, 40 and 60, respectively. The results indicated that electric field intensity and number of pulses both increased the consistency index of skim milk.

Table 6. 4 The electric field intensity and number of pulses effects on consistency index of skim milk.

Electric field intensity	P (n)		
(kV cm-1)	20	40	60
15	B 1.45 ± 0.03[a*]	AB 1.47 ± 0.02[a]	A 1.50 ± 0.03[a]
18	B 1.47 ± 0.02[a]	AB 1.50 ± 0.02[ab]	A 1.52 ± 0.02[a]
20	A 1.50 ± 0.03[a]	A 1.53± 0.04[a]	A 1.55 ± 0.03[a]

*Values with same letters in the row (a and b) are not significantly different ($P > 0.05$) and means with same letters in the column (A and B) are not significantly different ($P > 0.05$).

The effects of electric field intensity and number of pulses on the consistency index of reconstituted skim milk are shown in Figure 6.6. The consistency index is an indicator of the viscous nature of the solution. When the number of pulses was fixed (20 pulses) and the electric field intensity increased from 15 to 20 kV cm^{-1}, the consistency index of reconstituted skim milk increased from 1.45 to 1.50 (10^{-3} $Pa.s^n$). When the number of pulses was fixed (60 pulses) and the electric field intensity increased from 15 to 20 kV cm^{-1}, the consistency index of reconstituted skim milk increased from 1.50 to 1.55 (10^{-3} $Pa.s^n$). When the electric field intensity was fixed (18 kV cm^{-1}) and the number of pulses increased from 20 to 60 pulses, the consistency index of reconstituted skim milk increased from 1.47 to 1.52 (10^{-3} $Pa.s^n$). At the electric field intensity of 20 kV cm^{-1} and 60 pulses, the consistency index increased to the maximum value of 1.55 (10^{-3} $Pa.s^n$). Increases in the consistency index of skim milk with PEF treatments were significantly different ($P \leq 0.05$). These results confirmed that high electric field intensity had a strong effect on the consistency index of the reconstituted skim milk. A possible reason for an increase in the consistency index with PEF treatments could be that the protein denaturation and the increase size of casein micelles of milk were initiated during PEF

treatments. In fact, a visible coagulum appeared in skim milk. This confirmed that whey protein had partially denatured in skim milk with PEF treatments. Perez & Pilosof (2004) confirmed that the protein structure of β-lactoglobulin was partially denatured approximately 40% and the gelation rate was lowered when applying 10 pulses of 12.5 kV cm^{-1} for a treatment time on the order of millisecond.

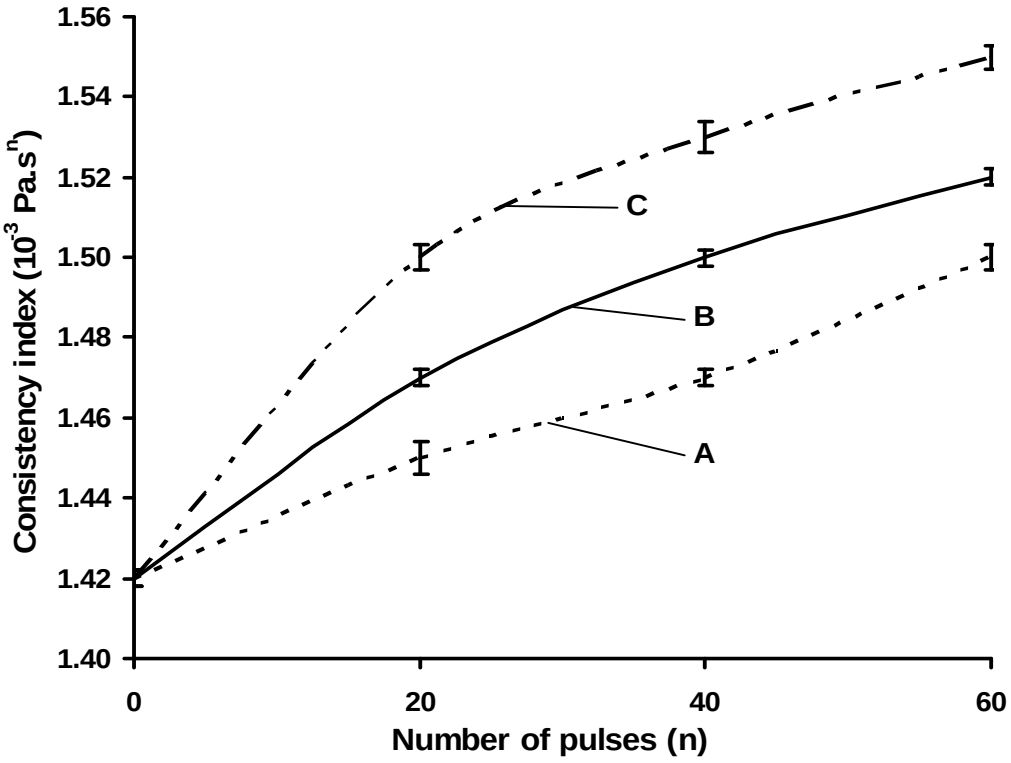

Figure 6. 6 Effect of electric field intensity and pulses on the consistency index of reconstituted skim milk. (A), 15 kV cm^{-1}; (B), 18 kV cm^{-1} and (C), 20 kV cm^{-1}.

6.6 CONCLUSION

The effects of PEF treatment on the flow behavior and viscosity of the reconstituted skim milk were studied. The PEF-treated skim milk samples followed the Herschel Bulkley model and exhibited shear-thinning behavior. PEF affected the rheological properties of skim milk and also affected the protein structural properties of

skim milk. PEF treatments increased the apparent viscosity of skim milk and the values were greater than the values of the control samples and the apparent viscosity increased to the maximum value of 4.25 (10^{-3} Pa.s) at the electric field intensity of 20 kV cm^{-1} and 60 pulses. When the electric field intensity increased from 15 to 20 kV cm^{-1} and the number of pulses increased from 20 to 60, the consistency index of skim milk increased from 1.45 to 1.55 (10^{-3} $Pa.s^n$). These results confirmed that electric field intensity and number of pulses both affected the flow behavior and apparent viscosity of skim milk. Further work is needed to assess the effect of PEF and temperature on the rheological properties of milk samples.

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CONNECTING TEXT

A comprehensive review of literature demonstrated the need for a study regarding the effects of pulsed electric field (PEF) on the physicochemical properties of food proteins. The following study sought to determine rheological and color properties of soy milk.

Chapter 7 describes how the rheological and color properties of soy milk were affected by PEF treatments and analyzed using rheometer and spectrophotometer. PEF treatments used different electric field intensities of 18, 20 and 22 kV cm^{-1} and number of pulses of 25, 50, 75 and 100. The results of this study confirmed that the changes of rheological properties and color properties of soy milk took place under these PEF conditions. The results confirmed that PEF treatments changed the rheological properties and color properties of soy milk.

7. PULSED ELECTRIC FIELD TREATMENTS EFFECTS ON RHEOLOGICAL AND COLOR PROPERTIES OF SOY MILK

7.1 ABSTRACT

The effects of pulsed electric fields (PEF) treatments on rheological and color properties of soy milk were studied. The flow behavior, viscosity and rheological parameters of PEF-treated soy milk were studied using a controlled stress rheometer. PEF treatments consisted of electric field intensity of 18, 20 and 22 kV cm^{-1}, number of pulses of 25, 50, 75 and 100, capacitance of 0.33 μF and pulse frequency of 0.5 Hz. Rheological properties at shear rates between 0 to 200 s^{-1} were measured for soy milk samples. The rheological behavior of the control and the PEF-treated soy milk were described using a power law model. The rheological properties of soy milk were affected by PEF treatments. The apparent viscosity of soy milk increased from 6.62 to 7.46 (10^{-3} Pa.s) with increasing electric field intensity from 18 to 22 kV cm^{-1} and pulses from 0 (the control sample) to 100. The consistency index (K) of soy milk also changed with PEF treatments. The color changes of soy milk with PEF treatments were measured using a spectrophotometer. Lightness (L^*), red/greenness (a^*), and yellowness/blueness (b^*) of soy milk were affected by PEF treatments.

Keywords: Soy milk, pulsed electric fields (PEF), rheological property, flow behavior, viscosity.

7.2 INTRODUCTION

Consumer awareness of food safety concerns requires the food industry to adopt new technologies for food preserving and food processing. The current trend is to produce food which has high quality, minimally processed and free from any additives. The rheological properties and color properties of liquid foods are very important for consumers since they mostly buy and enjoy foods based on these properties. Pulsed

electric fields (PEF) processing has been proposed as a non-thermal alternative method to inactivate microorganisms and keep the food long shelf life and also has its minimal effect on quality, sensory and nutritional attributes (Barsotti et al., 2002; Rastogi, 2003; Perez & Pilosof, 2004). Most PEF research has been related to inactivation of microorganisms and enzymes in liquid foods (Qin et al., 1995; Qin et al., 1996; Vega-Mercado et al., 1995; Vega-Mercado et al., 1997; Pothakamury et al., 1996; Ho et al., 1997; PureBright, 1998; Barsotti & Cheftel, 1999; Wouters et al., 1999; Yeom et al., 1999; Barbosa-Cánovas et al., 1997; Wouters & Smelt, 1997). The level of inactivation achieved with PEF treatments depends mainly on electric field intensity and number of pulses during PEF processes (Qin et al., 1995; Pothakamury et al., 1996; Martín et al., 1997; Bendicho et al., 2002).

Soy milk is a very popular liquid food in Asian countries, especially China and Japan. Almost all nutrient components such as protein, carbohydrates and lipids are present in soy milk (Guo et al., 1997). Thermal treatment is normally used to process soy milk. It is well known that thermal treatments induce dissociation, denaturation and aggregation of soy protein (Zhang et al., 2005). It is necessary to develop an alternative method to produce nutritious soy milk with minimum changes in color, quality and flavor.

The rheological properties of food products are very useful for product development, quality control, sensory evaluation and design and evaluation of the process equipment (Ahmed et al., 2003). The role of protein structure on the rheology of emulsions and gel is a complex one, and fundamental rheological tests provide critical information on time-dependent viscoelastic behavior and the changes in structure when a protein undergoes gelation or denaturation (Phillip et al., 1994). The rheological properties of proteins are influenced by temperature, concentration and the physical state of dispersion (Van Vliet & Walstra, 1980). For soy milk, heat treatments induce the denaturation of soy proteins mostly containing 7S globulin (β-conglycinin) and 11S globulin (glycinin) since denaturation might be involved in hydrophobic interactions of 7S globulin and the rupture of disulfide bonds of 11S globulin in soy protein (Zhang et al., 2004; Zhang et al., 2005). Also the viscosity of soy milk was increased with

increasing pressure treatment (Zhang et al., 2005) and heat treatment (Yamauchi et al., 1991). The effect of PEF on soy milk has been studied to evaluate microbial and enzyme inactivation in soy milk (Li et al., 2003; Li et al., 2006). There has not been any work on the effect of PEF treatments on the rheological properties and color properties of soy milk. Therefore, it would be very interesting to study these properties of soy milk as a function of PEF treatments.

In this study, the effects of PEF treatments on soy milk were studied using rheological and colorimetric measurements. The specific objective was to study rheological and color properties of soy milk after it had been exposed to PEF treatments.

7.3 MATERIALS AND METHODS

7.3.1 Sample preparation

Soy milk sample containing 3.2% soy protein, 2% fat and 4.8% carbohydrates (contents specified by the manufacturer) was purchased from a local grocery store. The soy milk was stored at 4^{o}C. The soy milk was transferred to room temperature for 2-3 hr such that the temperature of soy milk reached ~25^{o}C before PEF treatments.

7.3.2 Pulsed electric field treatments

PEF treatments were applied using a high voltage pulse generator TG2 (Food Process Engineering Laboratory, Macdonald Campus of McGill University) as described in Chapter 3. The soy milk sample was placed in a parallel plate treatment chamber (TC-01001). The soy milk samples were treated by PEF with electric field intensities of 18, 20 and 22 kV cm^{-1}, number of pulses of 25, 50, 75 and 100, capacitance from the discharge capacitor of 0.33 μF and pulse frequency of 0.5 Hz. The sample temperature of the treatment chamber was maintained below 26^{o}C by circulating 10^{o}C cold water from a water bath (W13, HAAKE Technik GmbH, Germany). The PEF-treated soy milk samples were immediately transferred to a refrigerator at 4^{o}C and the rheological and color properties of soy milk were evaluated within 2-4 hr.

7.3.3 Rheological measurements

Rheological measurements of soy milk sample were carried out using a controlled stress rheometer (AR 2000, TA Instruments, New Castle, DE, USA) with attached computer software (Rheology Advantage Data Analysis, TA version 5.1). The rheological measurements were carried out according to the methods as described in chapter 6. Apparent viscosity has many useful applications in characterizing a fluid food; especially in the characterization of shear-thinning fluids, the apparent viscosity is a useful parameter (Rao, 2000). The apparent viscosity of soy milk with PEF treatments was computed at the low shear rate of 10 s^{-1}. The low shear rate of 10 s^{-1} was selected because it has been reported as an effective shear rate (Morris, 1983).

The TA instrument software (Rheology Advantage, TA version 5.1) was used to conduct all data analysis. The rheological parameters were estimated by model fitting to minimize the standard error (Kristensen et al., 1996). The choice of a rheological flow model was based on evaluation of standard error and regression values that fit the models. The model that best fits for the control and the PEF-treated soy milk was selected.

7.3.4 Color measurements

Color measurements were made by using a Minolta spectrophotometer Model CM-3500d (Minolta Co., Ltd, Osaka, Japan). The color measurement was done by using Hunter color values, namely L^*, a^* and b^*. The lightness coefficient, L^*, ranges from black = 0 to white = 100. The coordinates (a^*, b^*) locate the color on a rectangular coordinate grid perpendicular to the L^* axis. a^* values indicate red-purple (positive values) and bluish-green (negative value). The vertical axis represent b* values, which positive b* value indicates yellow and negative b^* indicates blue. An increase in L^* value represents an increasing in whiteness or lightness. An increase in a^* value represents an increasing in redness or greenness (for negative value) and an increase in b^* value indicates an increasing in yellowness. Before each measurement the instrument was calibrated with a white standard tile (L = 96.90, a = -0.12, b = -0.21). A glass Petri-dish

containing about 3 mL of PEF-treated soy milk sample was placed above the light source and *L*, a** and *b** values were determined.

7.4 STATISTICAL ANALYSIS

All experiments were conducted with three replicates; means and standard deviations were reported. Analysis of variance (ANOVA) was undertaken with PROC GLM in SAS software (SAS 9.1, SAS Institute Inc., Cary, NC, USA) for all data analysis.

7.5 RESULTS AND DISCUSSION

7.5.1 Flow models

Various rheological models (Newtonian, Bingham, Casson, Power law and Herschel Bulkley) were evaluated for shear stress and shear rate data of the control and PEF-treated samples. Flow curves (shear stress compared to shear rate) were determined at increasing and decreasing shear rates between 0 and 200 s^{-1} in 10 minutes (up and down flow curves). The flow behavior of soy milk was determined by the flow model based on the standard error. The power law model shown as the equation (1) described the flow behavior of the soy milk samples.

$$\tau = K(\gamma)^n, \quad (1)$$

Where, τ is shear stress (Pa); γ is shear rate (s^{-1}); K is consistency index ($Pa.s^n$); n is flow behavior index (dimensionless) (n<1 for a shear-thinning fluid and n=1 for a Newtonian fluid). The shear stress and shear rate data fitted the power law model in all PEF treatment cases. The PEF-treated sample also clearly showed the shear-thinning behavior (n = 0.85-0.99, $R \leq$ >0.05). Here Table 7.1 shows the fitting of various flow models for soy milk at PEF treatments of electric field intensity of 22 kV cm^{-1} and 75 pulses.

Table 7. 1 Fitting of flow models for PEF-treated soy milk at electric field intensity of 22 kV cm^{-1} and 75 pulses.

Model	Consistency index, (K), (10^{-3} Pa.s^n)	Flow behavior index, (n)	Standard error
Newtonian	3.93	/	33.32
Bingham	3.38	/	4.45
Casson	2.91	/	2.34
Power law	7.88	0.852	0.82
Herschel-Bulkley	3.95	0.971	3.92

7.5.2 Effect of PEF on rheological behaviors of soy milk

Shear stress was plotted against shear rate to examine the flow behavior of soy milk with PEF treatments. Figure 7.1 shows the shear stress and shear rate for the control and the PEF-treated soy milk. PEF treatment resulted in an increase in shear stress at any shear rate and shear stress increased with PEF treatment (at 22 kV cm^{-1} and 75 pulses). The shear stress of the PEF-treated soy milk was found to be significantly affected by PEF treatments and shear rate ($P \leq 0.05$). The results confirmed that the higher electric field intensity had greater effect on the shear stress of soy milk.

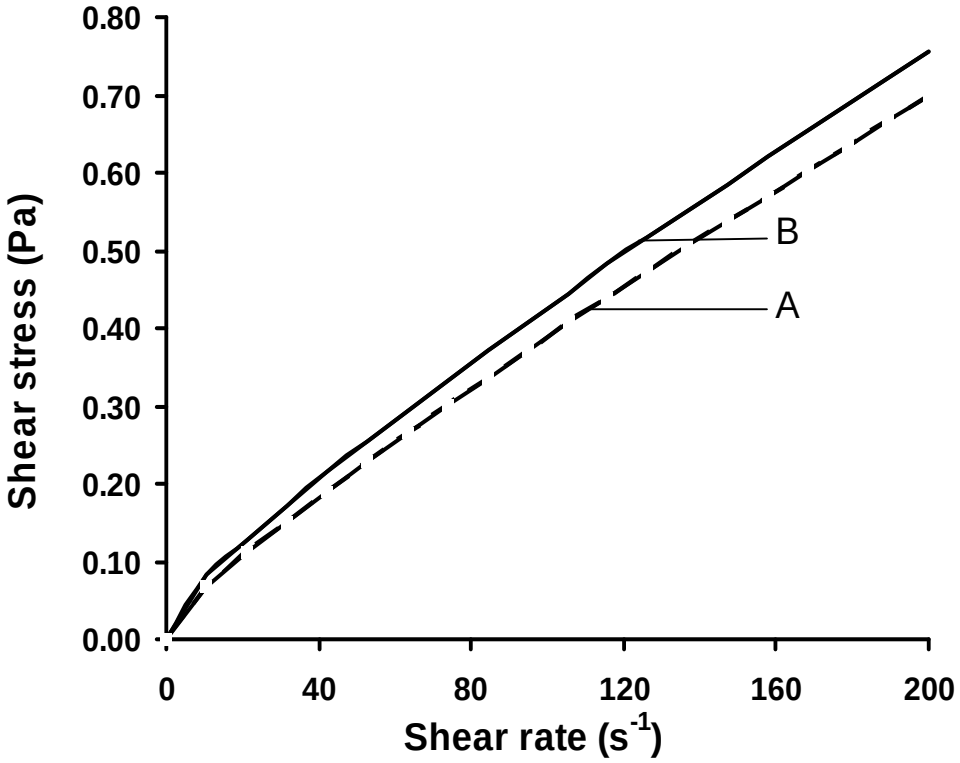

Figure 7. 1 Comparation of the shear stress versus shear rate plot of soy milk. (A), Control and (B), PEF-treated soy milk at 22 kV cm^{-1} and 75 pulses.

The electric field intensity (18, 20 and 22 kV cm^{-1}) and number of pulses (25, 50, 75 and 100) affected the apparent viscosity of soy milk as shown in Table 7.2. All PEF treatments resulted in increases in the apparent viscosity of soy milk and all obtained values were significantly ($P \leq 0.05$) greater than that of the control (6.62 ± 0.06 (10^{-3} Pa.s)). Increases in the apparent viscosity of PEF-treated soy milk were significantly different ($P \leq 0.05$) with increasing electric field intensity and number of pulses. The results indicated that electric field intensity and number of pulses both increased the apparent viscosity of soy milk.

Table 7. 2 The electric field intensity and number of pulses effects on apparent viscosity of soy milk.

Number of pulses (n)	Electric field intensity (kV cm^{-1})		
	18	20	22
25	D 6.72^{c}*	D 6.84^{b}	D 6.87^{a}
50	C 6.93^{c}	C 7.04^{b}	C 7.14^{a}
75	B 7.02^{c}	B 7.13^{b}	B 7.24^{a}
100	A 7.20^{c}	A 7.33^{b}	A 7.46^{a}

*Values with same letters in the row (a, b and c) are not significantly different ($P > 0.05$) and means with same letters in the column (A, B, C and D) are not significantly different ($P > 0.05$), standard deviation for each value was not shown here.

The apparent viscosities versus the shear rates of PEF treated soy milk are shown in Figure 7.2. The apparent viscosity of soy milk showed a considerable decrease with an increasing the shear rate from 0 to 200 s^{-1} when electric field intensity increased from 20 to 22 kV cm^{-1}. The apparent viscosity of the control sample decreased from 5.65 to 3.51 (10^{-3} Pa.s) with increasing shear rate from 0 to 200 s^{-1}. The apparent viscosity of the PEF-treated soy milk decreased from 7.82 to 3.79 (10^{-3} Pa.s) with increasing shear rate from 0 to 200 s^{-1} at 22 kV cm^{-1}. The effect of shear rate on the apparent viscosity of soy milk was significantly affected by PEF treatments ($P \leq 0.05$). Soy milk with PEF treatments showed the power law model flow behavior with quite low viscosity ($< 10 \times 10^{-3}$ Pa.s) and there is no interaction between particles. The results were similar to soy protein emulsions with homogenizing pressure treatments (Floury et al., 2002). A rapid breakdown of the structure occurs on initial shearing followed by much slower changes at higher shear rates. The behavior can be attributed to combined effects of breakdown of weak linkages between the proteins and reformation of such linkages as a result of Brownian motion and molecular collisions (Tang et al., 1993). A possible reason for a

decrease in apparent viscosity with increasing shear rate could be the fact that the soy protein coagulation and fat globule size changed and distribution in soy milk were initiated during PEF treatments. To ascertain the exact nature of flow behavior of soy milk, further study would be necessary to examine the characteristics of the constituent proteins, fats, carbohydrates, and their complexes, and how they might change with PEF. The same results were confirmed by Saif et al. (2004) for caprine milk with increasing temperature and shear rate.

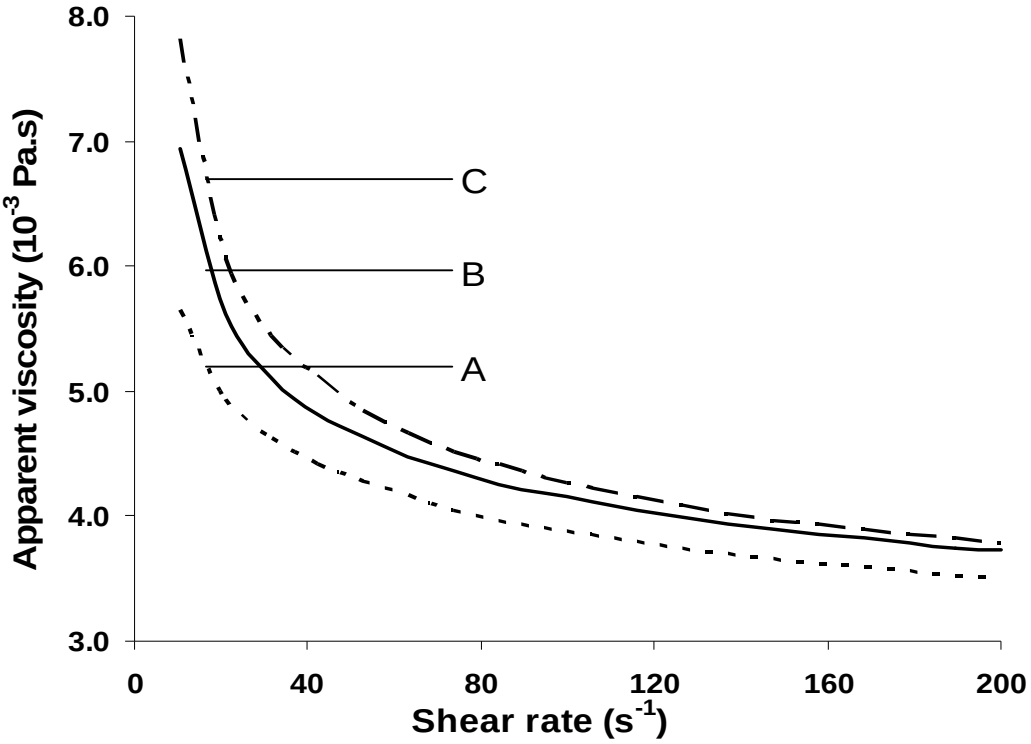

Figure 7. 2 Apparent viscosity of soy milk as a function of shear rate with PEF treatments at 75 pulses. (A), Control; (B), 20 kV cm^{-1} and (C), 22 kV cm^{-1}.

The apparent viscosities of soy milk versus PEF treatments are shown in Figure 7.3. The apparent viscosities of the soy milk samples were measured at the low constant shear rate of 10 s^{-1}. When electric field intensity increased from 18 to 22 kV cm^{-1}, the

apparent viscosity of soy milk increased from 6.62 to 7.46 (10^{-3} Pa.s). The apparent viscosity of soy milk increased from 6.62 to 7.32 (10^{-3} Pa.s) with increasing number of pulses ranging from 0 to 100 at an electric field intensity of 20 kV cm^{-1}. The apparent viscosity of soy milk showed a considerable increase with increasing electric field intensity and number of pulses. Effect of number of pulses on the apparent viscosity of soy milk was significant ($P \leq 0.05$) with increasing electric field intensity. Floury et al. (2002) obtained the similar results with soy protein emulsion with homogenized pressure treatment. The same phenomenon happened with heat-treated soy milk: soy protein dispersion increased the apparent viscosity after heating and underwent an irreversible change to the pre-gel state (Yamauchi et al., 1991). A similar result was obtained with using high pressure treatments with soy milk (Zhang et al., 2005). In soy milk, an increase in apparent viscosity with PEF treatments was supposed to reflect intermolecular interactions resulting from the attractions between adjacent denatured molecules, with the formation of weak transient networks. These interactions would cause an increased effective volume. As the protein aggregation became larger, they occupied more space and thus contributed to an increase in the apparent viscosity of a fluid system (Adapa et al., 1997).

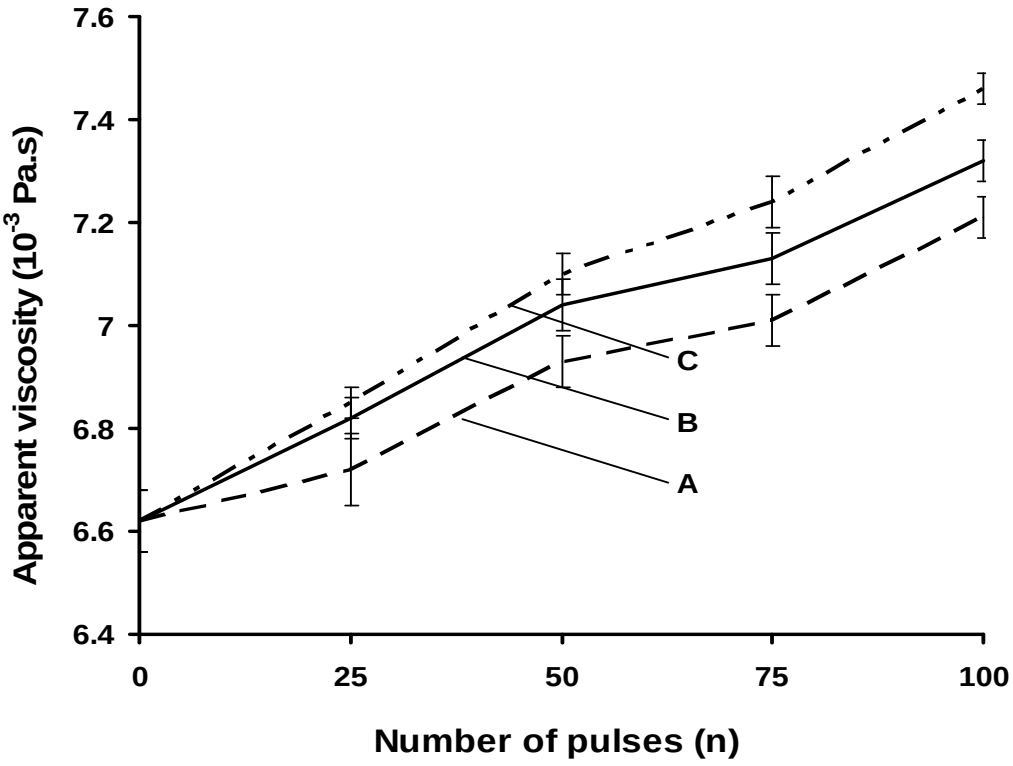

Figure 7. 3 Effects of electric field intensity and number of pulses on the apparent viscosity of soy milk. (A), 18 kV cm^{-1}; (B), 20 kV cm^{-1} and (C), 22 kV cm^{-1}.

The electric field intensity (18, 20 and 22 kV cm^{-1}) and number of pulses (25, 50, 75 and 100) affected the consistency index of soy milk as shown in Table 7.3. All PEF treatments resulted in increases in the consistency index of soy milk and all obtained values were significantly ($P \leq 0.05$) greater than that of the control (8.65 ± 0.03 (10^{-3} $Pa.s^n$)). Increases in the consistency index of PEF-treated soy milk were significantly different ($P \leq 0.05$). The results indicated that electric field intensity and number of pulses both increased the consistency index of soy milk.

Table 7. 3 The electric field intensity and number of pulses effect on consistency index of soy milk.

Number of pulses (n)	Electric field intensity (kV cm^{-1})		
	18	20	22
25	B 7.78[a]*	D 7.43[c]	D 7.73[b]
50	D 7.51[c]	C 7.62[b]	C 8.03[a]
75	C 7.62[c]	B 7.87[b]	B 8.32[a]
100	A 7.84[c]	A 8.31[b]	A 8.51[a]

*Values with same letters in the row (a, b and c) are not significantly different ($P > 0.05$) and means with same letters in the column (A, B, C and D) are not significantly different ($P > 0.05$), standard deviation for each value was not shown here.

The consistency index of soy milk as a function of pulses with increasing electric field intensity (18 to 22 kV cm^{-1}) is shown in Figure 7.4. The consistency index is an indicator of the viscous nature of the solution. The magnitude of the consistency index of the control was 8.65 (10^{-3} $Pa.s^n$). The consistency index of soy milk increased from 8.65 to 9.15 (10^{-3} $Pa.s^n$) with increasing electric field intensity ranging from 18 to 22 kV cm^{-1}. The consistency index increased from 8.65 to 8.99 (10^{-3} $Pa.s^n$) at electric field intensity of 18 kV cm^{-1} and pulses ranging from 0 to 100, while the values increased from 8.65 to 9.15 (10^{-3} $Pa.s^n$) at 22 kV cm^{-1} and number of pulses ranging from 0 (the control) to 100. Effect of number of pulses on the consistency index of soy milk was significant ($P \leq 0.05$) with increasing the electric field intensity. This was confirmed that higher electric field intensity had a strong effect on the consistency index of soy milk. This result also confirmed that number of pulses affected on the consistency index of soy milk. Since visible coagulum appeared in soy milk after PEF treatments, a possible reason for an increase in the consistency index with PEF treatments could be that the protein denaturation and increased size of soy protein were initiated by PEF treatments. This

confirmed that soy protein partially denatured in soy milk with PEF treatments. A similar result was confirmed by Zhang et al. (2005) in soy milk with high pressure treatment.

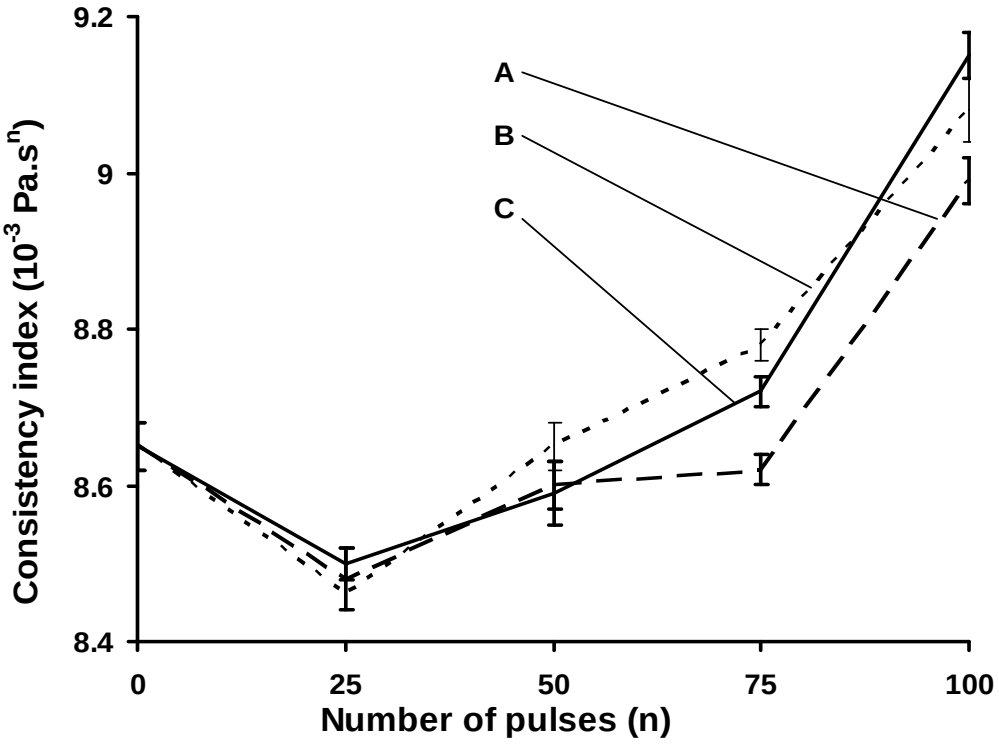

Figure 7. 4 Effects of electric field intensity and number of pulses on the consistency index of soy milk, (A), 18 kV cm^{-1}; (B), 20 kV cm^{-1} and (C), 22 kV cm^{-1}.

7.5.3 Color changes of PEF-treated soy milk

The electric field intensity (18, 20 and 22 kV cm^{-1}) number of pulses (25, 50, 75 and 100) had an effect on L^* value of soy milk as shown in Table 7.4. All PEF treatments resulted in changes in the colour L^* value of soy milk and all obtained values were significantly ($P \leq 0.05$) different than that of the control (81.31 ± 0.08).

Table 7. 4 The electric field intensity and number of pulses effect on color *L** value of soy milk.

Number of pulses (n)	Electric field intensity (kV cm^{-1})		
	18	20	22
25	A81.37[a]*	A81.24[b]	A81.29[b]
50	AB81.32[a]	B81.18[b]	B81.10[c]
75	BC81.27[a]	BC81.13[b]	B81.09[c]
100	C81.25[a]	C81.08[b]	C81.00[b]

*Values with same letters in the row (a, b and c) are not significantly different ($P > 0.05$) and means with same letters in the column (A, B and C) are not significantly different ($P > 0.05$), standard deviation for each value was not shown here.

Color changes of PEF treated soy milk were evaluated by using Minolta spectrophotometer and L^*, a^*, and b^* value represented the color change of soy milk. Figure 7.5 shows *L** value for soy milk with PEF treatments. *L** measures the change of whiteness and blackness between soy milk samples. There was a decrease in the *L** values when soy milk was PEF-treated with increasing electric field intensity and number of pulses. Effect of pulses on color *L* value* was significant ($P \leq 0.05$). This decrease in *L** value indicated the decreases in the whiteness of soy milk with PEF treatments.

The *a** value measures the differences of the red and green color components of soy milk. In Figure 7.5, there is a slight increase in *a** value with increasing electric field intensity from 18 to 22 kV cm^{-1}. But the effect of number of pulses on color *a** value was not significant ($P>0.05$). It showed that the soy milk was a little redder with PEF treatments. The *b** value measures the differences of the yellow and blue color components. Figure 7.5 shows a decrease in *b** value (toward blue) once soy milk was treated by PEF. The effect of pulses on *b** value was significant ($P \leq 0.05$). In soy milk,

PEF treatments produced darker (lower L^* value), redder (higher a^* value), and more yellow (higher b^* value) colors.

7.6 CONCLUSION

The effects of PEF treatments on the rheological properties of soy milk were studied. The PEF-treated soy milk samples followed the power law model. When electric field intensity increased from 18 to 22 kV cm^{-1}, the apparent viscosity of soy milk increased from 6.62 to 7.46 (10^{-3} Pa.s). As number of pulses increased from 0 (the control) to 100, the apparent viscosity of soy milk also increased from 6.62 to 7.32 (10^{-3} Pa.s) at electric field intensity of 20 kV cm^{-1}. The consistency index of soy milk increased from 8.65 to 9.15 (10^{-3} $Pa.s^n$) when electric field intensity increased from 18 to 22 kV cm^{-1} and number of pulses increased from 0 to 100. These results confirmed that electric field intensity and number of pulses affected the rheological properties of soy milk. The color changes (L^* and b^* values) of soy milk with PEF treatments were significant.

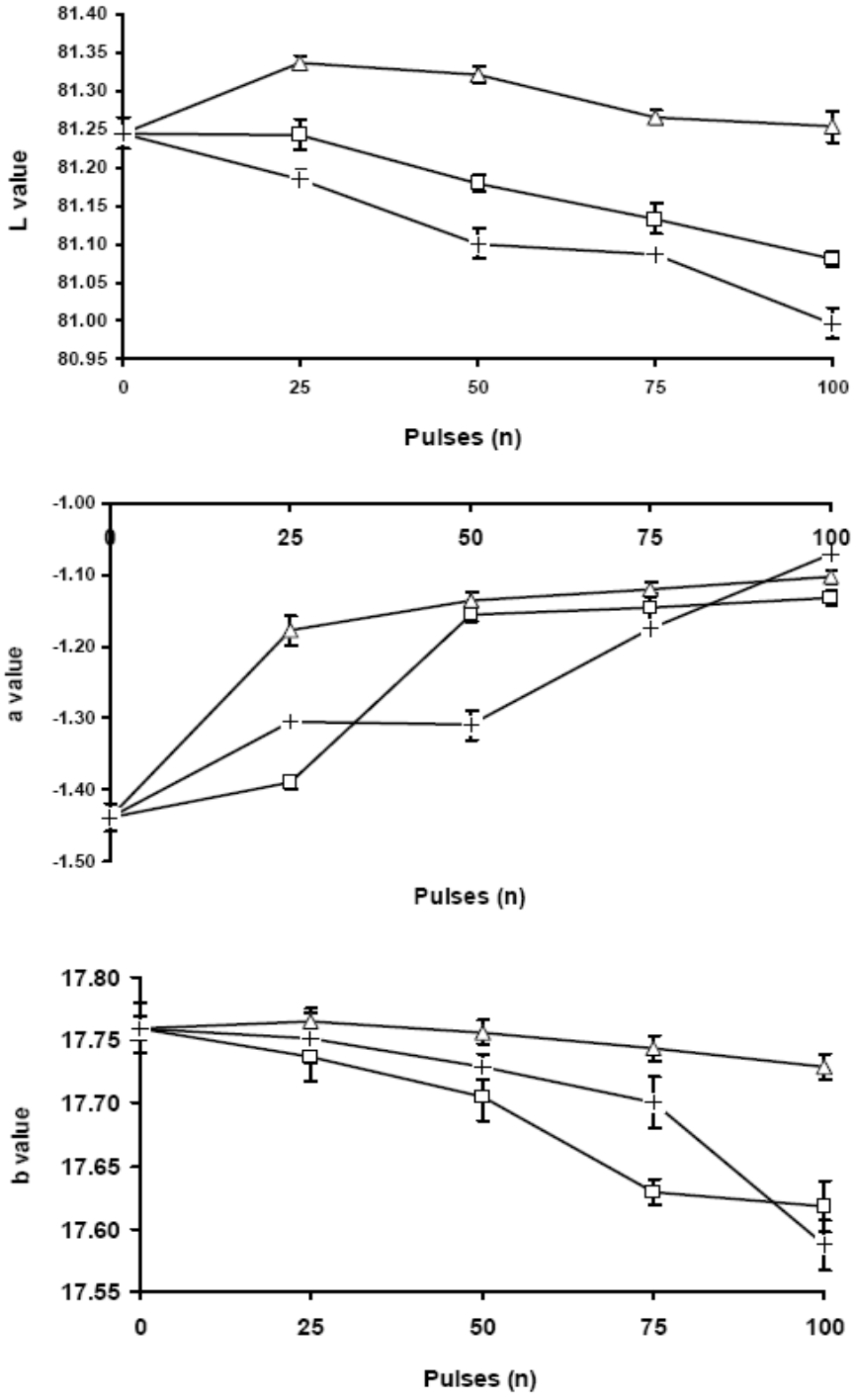

Figure 7. 5 Hunter *L**, *a** and *b** changes of soy milk with PEF treatments, (Δ), 18 kV cm^{-1}, (□), 20 kV cm^{-1} and (+), 22 kV cm^{-1}.

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8. GENERAL SUMMARY AND RECOMMENDATIONS FOR FUTURE RESEARCH

8.1 GENERAL SUMMARY

Over ten years of studies on pulsed electric fields (PEF) processing applied to food have shown that PEF processing could inactivate spoilage and pathogenic microorganisms and some enzymes in foods, while minimizing changes in the physical, nutritional and organoleptic qualities of the food, such as those observed under conventional heat treatments. Compared with the traditional heat treatments, PEF can cause minimum or no protein denaturation when it is used to inactivate microorganisms and give foods the long shelf life. Generally, heat treatments can make protein structural modification and change protein functional properties. There are other methods to make protein modification and improve functional properties for some food proteins such as physical modification, chemical modification, enzymatic modification and high pressure modification. If we want to improve food protein functional properties and we may make protein structural modification or change their rheological properties by using PEF treatments it must be done under special conditions (high pulse width and suitable electric field intensity). It is essential to explore protein modification brought upon by PEF treatments. Through this study, we understood how PEF treatments affected protein structural modification and altered rheological properties, thus ensured these food proteins the desired functional properties.

The overall objective of this study was to develop PEF treatments to replace or supplement heat treatments or other modification methods to make food protein structural modification and improve protein functional properties in food systems. In this regard, it was necessary to elucidate the main PEF parameters that are essential for designing and optimizing PEF conditions.

This study consisted of five sets of experiments which sought to determine: (*i*) the protein structural modification of whey protein at different electric field intensities and

number of pulses; (*ii*) the protein structural modification, surface hydrophobicity, thermal properties of whey protein after exposure to different electric field intensities and number of pulses; (*iii*) the protein structural modification and surface hydrophobicity of soy protein after exposure to different electric field intensities and number of pulses; (iv) the rheological behavior and rheological properties of reconstituted skim milk after exposure to different electric field intensities and number of pulses; and (v) the rheological properties and color property of soy milk after exposure to different electric field intensities and number of pulses.

The protein structural modification, thermal properties and rheological properties of selected food proteins were investigated by choosing different electric field intensities and number of pulses, and then the results confirmed by using fluorescence spectroscopy, differential scanning calorimetry (DSC) and controlled stress rheometer. The data of all studies were analyzed with Costat software, SAS software and Sigma Plot software to determine the significant differences obtained under different PEF treatment conditions.

The main conclusions and the major contributions to knowledge resulting from this research may be summarized below.

1. PEF treatments with different electric field intensities and number of pulses affected the intrinsic and extrinsic fluorescence intensities of WPI with different protein concentrations. The results proved that PEF treatments increased intrinsic fluorescence intensities and made red shifts at the emission wavelength and also increased extrinsic fluorescence intensities but made blue shifts at the emission wavelength. Overall, the results indicated that electric field intensity and number of pulses both modified protein structure of whey protein. Modification of protein structure of whey proteins resulted in the exposure of more hydrophobic regions and which increased the surface hydrophobicity of WPI.

2. The effects of PEF on structural modification and thermal properties of WPI were studied. The increases in the intrinsic fluorescence intensity of WPI and a red shift of 2-4 nm of the emission wavelength of the maximum fluorescence intensity were observed after PEF treatments. The extrinsic fluorescence intensities of whey proteins

also increased and blue shifts were made with PEF treatments. The thermal properties of whey protein were also changed with PEF treatments. The results suggested that the electric field intensity and number of pulses were closely related to their effects on structural modification and thermal properties of WPI. Comparison of DSC thermal parameters of whey protein showed that the thermal stability of whey protein was greatly reduced by PEF treatments. Further studies on the effect of PEF treatments on the whey protein are needed to understand its denaturation mechanism.

3. The present results indicated that electric field intensity and number of pulses both partially modified protein structure of SPI. The intrinsic fluorescence intensity of SPI increased and red shift of 2-6 nm in the emission wavelength were produced after PEF treatments. The increases in the intrinsic fluorescence intensity of SPI were related to the structural modification of soy protein with PEF treatments. The surface hydrophobicity of SPI increased with increasing electric field intensity or number of pulses. The results confirmed that the main parameters influencing structural modification of soy protein were the electric field intensity and number of pulses.

4. The PEF-treated skim milk samples' rheological properties followed the Herschel Bulkley model and exhibited shear-thinning behavior. PEF treatments (different electric field intensities and number of pulses) greatly influenced the rheological properties of the reconstituted skim milk. When electric field intensity increased, the apparent viscosity of reconstituted skim milk increased. The increase in the electric field intensity and number of pulses increased the consistency index of reconstituted skim milk. These results confirmed that PEF treatments affected the flow behavior, increased the consistency index and the apparent viscosity of the reconstituted skim milk. Further work is needed on PEF and temperature effects on the rheological properties of milk samples.

5. The PEF-treated soy milk samples' rheological properties followed the power law model. PEF treatments (different electric field intensities and number of pulses) greatly influenced the rheological properties of soy milk. These results confirmed that electric field intensity and number of pulses both affected the rheological properties of

soy milk. The color properties of soy milk treated with PEF also changed. PEF treatments significantly influenced the flow behavior, increased the apparent viscosity and the consistency index of soy milk.

8.2 RECOMMENDATIONS FOR FUTURE RESEARCH

Further investigation is required to study structural modification, thermal properties, rheological properties of food proteins as affected by different PEF conditions. Especially, it is required to study different PEF parameters (electric field intensity, pulse width, number of pulses, capacitance, pulse waveform and different treatment chambers), temperature and protein concentrations with respect to how they affect structural modifications, thermal properties, rheological properties of food proteins. In addition, the functional properties of PEF-treated food proteins, such as solubility, emulsifying properties, foaming properties, water holding capacity, gelling properties and other functional properties should be further studied. Other methods to study protein structural modification, such as Fourier transform infrared (FTIR) spectroscopy, UV spectroscopy, polyacrylamide gel electrophoresis (PAGE), and scanning electron microscopy (SEM) should be investigated.

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APPENDICES

Appendix I: Assessment of conditions for ANOVA for WPI solution

	Intrinsic fluorescence intensity				Extrinsic fluorescence intensity			
	Normality			Homogeneity of variance	Normality			Homogeneity of variance
	Kurt	Skew	d'A-P	Bartlett X^2	Kurt	Skew	d'A-P	Bartlett X^2
Value	-0.38	-0.16	0.90	32.08	-1.25	-0.07	2.92	10.86
P value	0.55	0.61	0.64	0.015	0.05	0.82	0.23	0.86
Signif.	ns	ns	ns	*	ns	ns	ns	ns

Kurt. = kurtosis; Skew. = skewness; d'A-P = D'Agostino-Pearson K^2 test; Bartlett X^2 = Bartlett's test of the homogeneity of variances; ns = not significant ($P > 0.05$); * = significant ($P \leq 0.05$).

Appendix II: Analyses of variance (ANOVA) for effects of electric field intensity, number of pulses and protein concentration on the intrinsic fluorescence intensity of WPI.

Factors	d.f	F	*P*	Significant difference
Model	17	41.1	<0.0001	***
C	1	244.2	<0.0001	***
E	2	48.8	<0.0001	***
P	2	164.1	<0.0001	***
C × E	2	2.40	0.1051	ns
C × P	2	3.80	0.0319	*
E × P	4	2.62	0.0508	ns
C × E × P	4	1.40	0.2523	ns

*C = protein concentration of WPI solution (%); E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; ns = not significant ($P > 0.05$), * = significant ($P \leq 0.05$) and *** = highly significant ($P \leq 0.0001$).

Appendix III: Analyses of variance (ANOVA) for effects of electric field intensity, number of pulses and protein concentration on extrinsic fluorescence intensity of WPI.

Factors	d.f	F	*P*	Significant difference
Model	17	163.5	<0.0001	***
C	1	2119.5	<0.0001	***
E	2	172.4	<0.0001	***
P	2	148.1	<0.0001	***
C × E	2	1.65	0.2064	ns
C × P	2	2.56	0.0906	ns
E × P	4	2.07	0.1045	ns
C × E × P	4	0.36	0.8348	ns

*C = protein concentration of WPI solution (%); E = electric field intensity (kV cm^{-1}); N = number of pulses (n); d.f = degrees of freedom; ns = not significant ($P > 0.05$) and *** = highly significant ($P \leq 0.0001$).

Appendix IV: Assessment of conditions for ANOVA for WPI solution.

	Intrinsic fluorescence intensity (a.u.)				Extrinsic fluorescence intensity (a.u.)			
	Normality			Homogeneity of variance	Normality			Homogeneity of variance
	Kurt	Skew	d'A-P	Bartlett X^2	Kurt	Skew	d'A-P	Bartlett X^2
Value	0.24	0.66	2.50	23.27	-0.10	0.56	1.78	5.17
P value	0.78	0.14	0.29	0.003	0.91	0.21	0.41	0.74
Signif.	ns	ns	ns	*	ns	ns	ns	ns
	Denaturation temperature, T_{d1} (^oC)				Denaturation temperature T_{d2} (^oC)			
	Normality			Homogeneity of variance	Normality			Homogeneity of variance
	Kurt	Skew	d'A-P	Bartlett X^2	Kurt	Skew	d'A-P	Bartlett X^2
Value	-0.75	0.56	2.68	1.42	-1.07	0.48	2.84	3.49
P value	0.38	0.21	0.26	0.99	0.22	0.28	0.24	0.90
Signif.	ns	ns	ns	ns	ns	ns	ns	ns

	Enthalpy ΔH (J g^{-1})			
	Normality			Homogeneity of variance
	Kurt	Skew	d'A-P	Bartlett X^2
Value	-0.73	0.33	1.58	4.32
P value	0.40	0.46	0.45	0.83
Signif.	ns	ns	ns	ns

Kurt. = kurtosis; Skew. = skewness; d'A-P = D'Agostino-Pearson K^2 test; Bartlett X^2 = Bartlett's test of the homogeneity of variances; ns = not significant ($P > 0.05$), * = significant ($P \leq 0.05$).

Appendix V: Analyses of variance (ANOVA) for effects of electric field intensity and number of pulses on the intrinsic fluorescence intensity of WPI.

Factors	d.f	F	*P*	Significant difference
Model	8	50.5	<0.0001	***
E	2	71.4	<0.0001	***
P	2	109.9	<0.0001	***
E × P	4	10.4	0.0001	***

*E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; and *** = highly significant ($P \leq 0.0001$).

Appendix VI: Analyses of variance (ANOVA) for effects of electric field intensity and number of pulses on the extrinsic fluorescence intensity of WPI.

Factors	d.f	F	*P*	Significant difference
Model	8	73.7	<0.0001	***
E	2	151.4	<0.0001	***
P	2	138.4	<0.0001	***
E × P	4	2.6	0.0718	ns

*E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; *** = highly significant ($P \leq 0.0001$) and ns = not significant ($P > 0.05$).

Appendix VII: Analyses of variance (ANOVA) for effects of electric field intensity and number of pulses on denaturation temperature T_{d1} for WPI.

Factors	d.f.	F	*P*	Significant difference
Model	8	31.8	<0.0001	***
E	2	3.6	0.0489	*
P	2	96.3	<0.0001	***
E × P	4	13.6	<0.0001	***

*E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; * = significant ($P \leq 0.05$) and *** = highly significant ($P \leq 0.0001$).

Appendix VIII: Analyses of variance (ANOVA) for effects of electric field intensity and number of pulses on denaturation temperature T_{d2} for WPI.

Factors	d.f	F	*P*	Significant difference
Model	8	179.4	<0.0001	***
E	2	167.9	<0.0001	***
P	2	542.3	0.0001	***
E × P	4	3.7	0.0234	*

*E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; * = significant ($P \leq 0.05$) and *** = highly significant ($P \leq 0.0001$).

Appendix IX: Analyses of variance (ANOVA) for effects of electric field intensity and number of pulses on apparent enthalpy ΔH (J g^{-1}) for WPI solution.

Factors	d.f	F	*P*	Significant difference
Model	8	61.2	<0.0001	***
E	2	32.8	<0.0001	***
P	2	205.3	<0.0001	***
E × P	4	3.3	0.0324	*

*E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; * = significant ($P \leq 0.05$) and *** = highly significant ($P \leq 0.0001$).

Appendix X: Assessment of conditions for ANOVA for SPI solution

	Intrinsic fluorescence intensity (a.u.)				Extrinsic fluorescence intensity (a.u.)			
	Normality			Homogeneity of variance	Normality			Homogeneity of variance
	Kurt	Skew	d'A-P	Bartlett X^2	Kurt	Skew	d'A-P	Bartlett X^2
Value	-0.72	0.43	1.79	3.306	-0.86	0.46	2.15	2.37
P value	0.43	0.37	0.41	0.8553	0.35	0.33	0.34	0.94
Signif.	ns	ns	ns	ns	ns	ns	ns	ns

*Kurt. = kurtosis; Skew. = skewness; d'A-P = D'Agostino-Pearson K^2 test; Bartlett X^2 = Bartlett's test of the homogeneity of variances; ns = not significant ($P > 0.05$).

Appendix XI: Analyses of variance (ANOVA) for effects of electric field intensity and number of pulses on the intrinsic fluorescence intensity of SPI.

Factors	d.f	F	*P*	Significant difference
Model	7	159.6	<0.0001	***
E	1	145.2	<0.0001	***
P	3	316.6	<0.0001	***
E × P	3	7.3	0.0026	*

*E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; * = significant ($P \leq 0.05$) and *** = highly significant ($P \leq 0.0001$).

Appendix XII: Analyses of variance (ANOVA) for effects of electric field intensity and number of pulses on the extrinsic fluorescence intensity of SPI.

Factors	d.f	F	*P*	Significant difference
Model	7	136.7	<0.0001	***
E	1	122.6	<0.0001	***
P	3	272.6	<0.0001	***
E × P	3	5.4	0.0092	*

*E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; *** = highly significant ($P \leq 0.0001$) and * = significant ($P \leq 0.05$).

Appendix XIII: Assessment of conditions for ANOVA for skim milk

	Shear stress				Yield stress			
	Normality			Homogeneity of variance	Normality			Homogeneity of variance
	Kurt	Skew	d'A-P	Bartlett X^2	Kurt	Skew	d'A-P	Bartlett X^2
Value	-0.49	0.45	1.71	1.88	-0.63	-0.41	1.73	4.56
P value	0.57	0.31	0.43	0.98	0.47	0.36	0.42	0.80
Signif.	ns	ns	ns	ns	ns	ns	ns	ns

	Consistency index				Flow behavior			
	Normality			Homogeneity of variance	Normality			Homogeneity of variance
	Kurt	Skew	d'A-P	Bartlett X^2	Kurt	Skew	d'A-P	Bartlett X^2
Value	-0.71	0.18	1.12	3.71	-1.24	-0.11	2.00	1.82
P value	0.42	0.69	0.57	0.88	0.15	0.81	0.37	0.99
Signif.	ns	ns	ns	ns	ns	ns	ns	ns

	Apparent viscosity			
	Normality			Homogeneity of variance
	Kurt	Skew	d'A-P	Bartlett X^2
Value	-1.23	0.10	1.96	2.20
P value	0.16	0.82	0.37	0.97
Signif.	ns	ns	ns	ns

Kurt. = kurtosis; Skew. = skewness; d'A-P = D'Agostino-Pearson K^2 test; Bartlett X^2 = Bartlett's test of the homogeneity of variances; ns = not significant ($P > 0.05$).

Appendix XIV: Analyses of variance (ANOVA) for effects of electric field intensity and number of pulses on flow behavior of skim milk

Factors	d.f	F	*P*	Significant difference
Model	8	128.6	<0.0001	***
E	2	44.3	<0.0001	***
P	2	450.1	<0.0001	***
E × P	4	10.1	0.0002	***

* E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; *** = highly significant ($P \leq 0.0001$).

Appendix XV: Analyses of variance (ANOVA) for effects of electric field intensity and number of pulses on apparent viscosity of skim milk.

Factors	d.f	F	*P*	Significant difference
Model	8	19315.8	<0.0001	***
E	2	4940.1	<0.0001	***
P	2	70046.7	<0.0001	***
E × P	4	1138.2	<0.0001	***

* E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; *** = highly significant ($P \leq 0.0001$).

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Appendix XVI: Analyses of variance (ANOVA) for effects of electric field intensity and number of pulses on apparent viscosity of skim milk.

Factors	d.f	F	*P*	Significant difference
Model	8	35.7	<0.0001	***
E	2	75.3	<0.0001	***
P	2	66.7	<0.0001	***
E × P	4	0.4	0.8266	ns

*E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; ns = not significant ($P > 0.05$) and *** = highly significant ($P \leq 0.0001$).

Appendix XVII: Assessment of conditions for ANOVA for soy milk

	Shear stress				Consistency index			
	Normality			Homogeneity of variance	Normality			Homogeneity of variance
	Kurt	Skew	d'A-P	Bartlett X^2	Kurt	Skew	d'A-P	Bartlett X^2
Value	0.68	0.85	5.49	91.01	-1.02	0.31	2.44	5.03
P value	0.37	0.03	0.06	0.0001	0.19	0.43	0.29	0.93
Signif.	ns	*	ns	***	ns	ns	ns	ns

	Flow behaviour				Apparent viscosity			
	Normality			Homogeneity of variance	Normality			Homogeneity of variance
	Kurt	Skew	d'A-P	Bartlett X^2	Kurt	Skew	d'A-P	Bartlett X^2
Value	16.21	3.31	52.55	-	-0.69	0.11	1.03	3.22
P value	0.000	0.000	0.000	-	0.37	0.78	0.56	0.99
Signif.	***	***	***	-	ns	ns	ns	ns

	Color *L* *				Color *a**			
	Normality			Homogeneity of variance	Normality			Homogeneity of variance
	Kurt	Skew	d'A-P	Bartlett X^2	Kurt	Skew	d'A-P	Bartlett X^2
Value	-0.82	-0.10	1.41	3.72	-0.17	-0.87	0.46	7.94
P value	0.28	0.80	0.49	0.98	0.82	0.03	0.09	0.72
Signif.	ns	ns	ns	ns	ns	*	ns	ns

	Colour *b**			
	Normality			Homogeneity of variance
	Kurt	Skew	d'A-P	Bartlett X^2
Value	0.72	0.34	1.07	6.54
P value	0.35	0.39	0.38	0.83
Signif.	ns	*	ns	ns

Kurt. = kurtosis; Skew. = skewness; d'A-P = D'Agostino-Pearson K^2 test; Bartlett X^2 = Bartlett's test of the homogeneity of variances; ns = not significant ($P > 0.05$), *= significant ($P \leq 0.05$).

Appendix XVIII: Analyses of variance (ANOVA) for effects of electric field intensity and number of pulses on apparent viscosity of soy milk.

Factors	d.f	F	*P*	Significant difference
Model	11	1784.32	<0.0001	***
E	2	1598.18	<0.0001	***
P	3	5420.24	<0.0001	***
E × P	6	2842.42	<0.0001	***

E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; *** = highly significant ($P \leq 0.0001$).

Appendix XIX: Analyses of variance (ANOVA) for effects of electric field intensity and number of pulses on consistency index of soy milk.

Factors	d.f	F	*P*	Significant difference
Model	11	1801.1	<0.0001	***
E	2	3855.28	<0.0001	***
P	3	3889.67	<0.0001	***
E × P	6	72.09	<0.0001	***

E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; *** = highly significant ($P \leq 0.0001$).

Appendix XX: Analyses of variance (ANOVA) for effects of electric field intensity and number of pulses on colour L^* value of soy milk.

Factors	d.f	F	*P*	Significant difference
Model	11	29.21	<0.0001	***
E	2	84.83	<0.0001	***
P	3	43.30	<0.0001	***
E × P	6	3.63	<0.011	*

E = electric field intensity (kV cm^{-1}); P = number of pulses (n); d.f = degrees of freedom; * = significant ($P \leq 0.05$) and *** = highly significant ($P \leq 0.0001$).