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Effect of moderate electric field on structural and thermo-physical properties of sunflower protein and sodium caseinate

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Abstract

Moderate electric field (MEF) were employed to sunflower protein isolate (SPI) and sodium caseinate (CAS) to change their structural and thermo-physical properties with a non-thermal approach. MEF treatment reduced significantly the average particle size with noticeable changes in the uniformity of SPI and CAS solutions. A maximum 10% reduction in the surface tension was observed for 0.2% w/w SPI sample by applying 150 V for 20 seconds. This effect was less pronounced for CAS samples and for the protein concentration of 2% w/w. FTIR spectra provided an increased α -helix content for SPI inducing a bulkier, loosened and more hydrophilic structure which is unlikely for CAS. Intrinsic Fluorescence Spectroscopy revealed that MEF treatments changed the tertiary structures of SPI and CAS. Denaturation temperatures and enthalpies of the SPI and CAS samples were decreased by MEF treatments.

Keywords: moderate electric field, sunflower protein, sodium caseinate, protein structure.

1. 1. Introduction

Food proteins are the key structural entities in food material not only for their high nutritional value, but also due to their techno-functional properties such as gelation, emulsification, and foaming (Foegeding & Davis, 2011). Environmental concerns, sustainability and possibly high expense and potential hazards of biological diversity are the driving forces to seek and obtain alternative protein resources (Alemayehu, Bendevis, & Jacobsen, 2015; Pojić, Mišan, & Tiwari, 2018; Sá, Moreno, & Carciofi, 2020). Sunflower protein is recently being investigated in terms of its functional properties as a potential alternative for commercially used proteins in food systems (Gonzalez-Perez & Vereijken, 2007; Salgado, Molina Ortiz, Petruccielli, & Mauri, 2011). Utilization of de-oiled press cake after sunflower oil production is a promising phenomenon to obtain sustainable plant based protein (Pickardt et al., 2009; Weisz, Schneider, Schweiggert, Kammerer, & Carle, 2010). Comparisons for functional properties of sunflower protein isolate (SPI) and other well-known and versatile proteins like soy protein, egg white and/or whey protein isolates functionalities are contradictory: Some studies declared at least equal or better emulsification and foaming of SPI than soy protein isolates (Albe Slabi et al., 2020; Sosulski & Fleming, 1977). On the other hand, many studies showed how weak the functionality of SPI than that of other protein isolates (Gonzalez-Perez & Vereijken, 2007; Pickardt et al., 2011). Different observations could be related with different agricultural properties of sunflower seed, type of the raw material (kernel, seed or de-oiled press cake), presence of natural phenolic compounds as well as applied protein extraction protocol (Albe Slabi et al., 2020; Pickardt, Eisner, Kammerer, & Carle, 2015). If SPI is extracted from industrial de-oiled press cake, the obtained 11S globulins (helianthinin) will already be denatured due to solvent extraction followed by heat treatment during oil extraction (González-Pérez et al., 2002; Molina, Petruccielli, & Anon, 2004). Even native and de-phenolized SPI has slight solubilities and do not form gel structures when heated (Sanchez & Burgos, 1997). For thermal denaturation/unfolding of helianthinin, a heat treatment over 90 °C (for pH 7.0) should be applied which also indicates it's thermal stability (Molina et al., 2004). SPI has the potential to be used for its functional properties but requires consistent and improved functional behaviors for ubiquitous usage or industrial upscaling.

Proteins can be modified to obtain better functional properties by using physical, chemical or enzymatic methods (Mirmoghtadaie, Shojaee Aliabadi, & Hosseini, 2016; Molina

et al., 2004; Shah, Umesh, & Singhal, 2019; Sun-Waterhouse, Zhao, & Waterhouse, 2014). Among these, physical processing methods such as microwave treatment, ohmic heating, high pressure processing, gamma irradiation, ultrasonication and pulsed electric field treatment have potential to modify varying protein types with lower costs, easy operation conditions, less time and energy consumption and no waste production when compared with chemical and enzymatic techniques (Beilen & Li, 2002; Caruggi, Lucisano, Feyissa, Yazdi, & Mohammadifar, 2019; Huppertz, Fox, & Kelly, 2004; L. Jiang et al., 2014; Li, Chen, & Mo, 2007; Malik, Sharma, & Saini, 2017a; McKerchar et al., 2019; Messens, Van Camp, & Huyghebaert, 1997; Mirmoghtadaie, Kadivar, & Shahedi, 2009; Mirmoghtadaie et al., 2016; Molina et al., 2004; Shah et al., 2019; Sun-Waterhouse et al., 2014; Tapal & Tikku, 2018; Xiong et al., 2018).

Electro-technologies like ohmic heating (OH) are physical methods using electric energy as the driving force which is one of the most clean and sustainable energy sources. OH uses the voltages in moderate electric field (MEF) to provide a rapid and homogenous heat distribution throughout the mass (Kaur & Singh, 2016). Applied MEF during OH treatment also performs a putative non-thermal effect on the protein structure and has a synergetic effect with the increased temperature. However, the non-thermal effect of MEF is not fully clear despite being studied over a decade. It is considered that MEF has a non-thermal effect on protein's tertiary structure and effecting the conformation throughout the hydrogen and ionic bonds and hydrophobic interactions (Pereira, Souza, Cerqueira, Teixeira, & Vicente, 2010; Pereira, Teixeira, & Vicente, 2011; Rodrigues, Avelar, Vicente, Petersen, & Pereira, 2020).

Ito et al. (2014) used 10^3 V cm^{-1} and 50 Hz frequency AC (alternating current) MEF application during freezing process to protect the freshness of purple sea urchin. In another study, induction effect of AC MEF application (667 V cm^{-1} in amplitude and 50 Hz frequency) on enzymatic activity of trypsin enzyme on bovine serum albumin digestion was observed (Okumura et al., 2017). They treated 5 mL sample in a custom unit at 4°C for 72 h. Rodrigues et al. (2020), combined MEF strength (10 V cm^{-1} and 20/50 MHz) with thermal treatment ($20 - 90^\circ\text{C}$) to investigate thermal unfolding of β -lactoglobulin. Bekard & Dunstan (2014) applied oscillating MEF in a small quartz electro cell (1 cm) to change the tertiary structure of bovine serum albumin and lysozyme for pharmaceutical applications. They reported that the protein was unfolded when it was exposed to an electric strength ($78 - 500 \text{ V cm}^{-1}$ with an oscillating frequency -sine wave- between $10 - 500 \text{ Hz}$ during 1.5 and 3 h, at below 25°C) extremely lower than its interchain and/or intramolecular forces for long enough

times. Non-thermal effect of MEF treatment might affect the structure of heat resistant SPI as a key for better functional properties.

In this study, we evaluate the structural, thermal and physical changes of SPI compared to CAS when exposed to non-thermal MEF application.

2. Material and methods

2.1 Materials

Crude, industrially de-oiled sunflower cake was kindly donated by Olin Edirne Oil Company (Edirne, Turkey). Cake was oven dried at 45 °C and kept at 4 °C until the protein extraction day. The maximum storage time between storage and the final extraction was 2 months. Protein was isolated from the crude de-oiled cake with ultrasound assisted isoelectric precipitation method (Subaşı et al., 2020). For phenolic compounds removal, dry cake was washed with 60% (v/v) methanol during 8 h. Ultrasound treatment (Branson Digital Sonifier SFX 550, St. Louis, MO, USA) at 20 kHz (using 100% power of 550 W) with a 0.5-inch probe, at 30 °C for 10 min was applied to increase the extracted protein yield (Dabbour et al., 2018). The collected de-phenolized protein precipitates were freeze dried (Christ Beta 1-8 LSCplus, Osterode am Harz, Germany) and stored at 4 °C during the experiments. The final SPI contained 87% (w/w) crude protein. The characteristics of the SPI was indicated in Subaşı et al. (2020). Sodium caseinate (CAS) and imidazole buffer substance were purchased from Merck Co. (Darmstadt, Germany). All the other chemicals used were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 MEF application

Protein powders were dissolved in imidazole buffer (pH 7.0) at 2% (w/w) and dissolved overnight on magnetic stirrer. Electric conductivities of SPI and CAS solution in buffer were 2.42 mS cm⁻¹ and 2.98 mS cm⁻¹, respectively at 25 °C. An insulated polyethylene-polypropylene (W500 grade) chamber with two opposing titanium electrodes was used. The adjustable gap between the electrodes, those were attached to the inner surface of the chamber, was 3.9 cm and the width of the cell was 9.5 cm. The chamber has a lid made of the same insulated material with a perpendicular thermocouple attached. The maximum power supply of the equipment was 230 V cm⁻¹ using the 60 Hz sine wave alternating current. The system had a screen for temperature besides equipped with a voltmeter and an amperemeter as depicted in Fig. 1.

Application parameters were limited by the electric current transmitting capability of the equipment, except final temperature of the protein solution. Temperature of the sample was screened precisely and maximum allowed temperature was assigned as 45 °C in order to complete the process as “non-thermal”. All samples were at 25 °C prior to electric field treatments. Following this approach, the lowest and the highest applicable electro power were chosen. In this electric circuit, the protein solution was acting as the resistant element. The lowest detectable current by the amperemeter was 0.5 A, yielding 10 V cm⁻¹ electric field when transmitted through the protein solution. Two different application time were chosen to see the effect of treatment time. The lowest voltage long time electric field application parameters were adopted according to the study of Bekard & Dunstan (2014). The “v1” abbreviation was nominated for “sample applied 10 V cm⁻¹ for 1 h”. Similarly, “v2” was nominated for “sample applied 10 V cm⁻¹ for 2 h”. The final temperatures of the protein solutions after treatments reached to 38 °C and 45 °C, respectively. Similarly, a higher voltage with the most likely short and longer application times were determined. The shortest application time to cut off the current after switching on could be 5 sec. The voltage should also be applicable for the most likely long time as well as the 5 sec application. Some preliminary tests were performed to choose this higher voltage considering the temperature not to exceed 45 °C. Following these conditions, the voltage was determined as 150 V cm⁻¹. The “V5” abbreviation was nominated for “sample applied 150 V cm⁻¹ for 5 sec”. Similarly, “V20” was nominated for “sample applied 150 V cm⁻¹ for 20 sec”. The current, when 150 V cm⁻¹ electric field was applied to the protein sample was 6.7 A. The final temperature of the protein solutions after treatments reached to 30 °C and 45 °C, respectively. All treated samples were immediately cooled down to 25 °C in an ice bath.

In order to elucidate the effect of the thermal history, another sample group was prepared in the same volume, concentration and buffer. The control groups were heated to 30 °C, 38 °C and 45 °C using a water bath, the same temperature values with MEF treated samples reached up. They were immediately cooled down to 25 °C, in an ice bath. Particle size analysis and surface tension measurements were carried out on the control samples. No significant differences were detected (data not shown) between the native forms and control group proteins. It was assumed the heating effect until 45 °C for denaturation/unfolding of SPI and CAS is neglectable as expected.

Fig. 1. Chamber of MEF treatment. → and ← represent the directions of electric field (action) and drag force (reaction) affecting the protein particles. → is the potential change in protein particle, when exposed to electric field and drag forces. V, A, T and t represent voltmeter, amperemeter, temperature monitor and timer, respectively.

2.3 Characterization of the samples

2.3.1. Particle size distribution

Particle size distribution and polydispersity index (PdI) were measured by via Zetasizer Nano ZS (Malvern Instruments, Ltd., UK). All protein samples were dissolved in imidazole buffer solution (pH 7.0) with a final protein concentration of 0.004% (w/w). No further filtration was applied. DTS-1070 disposable folded capillary cells (Malvern Instruments, Ltd., UK) were used for measurements. Analysis were completed at 25 °C as triplicate.

2.3.2. Interfacial tension measurements

The interfacial tension at different concentrations was measured using a microtensiometer (Kibron EZPiplus, Helsinki, Finland) at 25 °C. The instrument was initially calibrated with deionized water. Per measurement, 3 mL of sample was used. The du Nouy

method was used to determine the surface tension by the equipment. All samples were prepared at different concentrations as 0.2, 0.5, 1.0, 1.5 and 2.0% in imidazole buffer (w/w, pH 7.0). Protein solutions were dissolved overnight for the assumption of reaching thermodynamic stability. Surface tension for deionized water was recorded during calibration as $72.4 \pm 0.2 \text{ mN m}^{-1}$ which was consistent with the literature (Adamson, 1990).

2.3.3. Fourier Transform Infrared Spectroscopy (FTIR) spectra

FTIR spectra was recorded in the transmission mode between the range of 4000 – 500 cm^{-1} with a Perkin-Elmer Spectrum 100 spectrometer (Waltham, Massachusetts, USA), based on a Universal Attenuated Total Reflectance sensor 125 (UATR-FTIR). Signals were recorded in every 4 scans and the spectra was plotted as the percentage transmittance (%T) as a function of wavenumber (cm^{-1}).

2.3.4. Intrinsic fluorescence spectroscopy

Intrinsic fluorescence measurements were done using the FS5 Spectrofluorometer (Edinburgh Instruments, Livingston, UK) with a 150 W Xenon lamp and a single photon counting photomultiplier (PMT) detector (Hamamatsu, R928P) with a voltage of 600 V. Slit width was 2 nm for both excitation and emission. Samples were excited (λ_{ex}) at 280 nm and the emission wavelength range was from 290 to 420 nm (with 2 nm measurement steps). Scan rate was 1200 nm min^{-1} . The protein solutions were prepared at 1 mg mL^{-1} and scanned in triplicate. The spectra were averaged to reduce the noise.

2.3.5. Differential scanning calorimetry (DSC)

Thermal properties were analyzed with the TA dynamic scanning calorimeter (DSC 250, TA Instruments, New Castle, Delaware, USA). The instrument was calibrated with indium ($T_{\text{peak}} = +156.5^\circ\text{C}$, $\Delta H_{\text{melting}} = 28.5 \text{ J g}^{-1}$) and sapphire and the cell purge was conducted with dry nitrogen (50 mL min^{-1}). Roundly 3.5 mg of samples (moisture content was around 10% w/w) were sealed in hermetic aluminum pans. Another identical empty pan was used as a reference. Temperature modulation was applied from -20 to 150°C with 5°C min^{-1} steps. No sample loss was observed. Each sample was run in triplicate.

2.4. Statistical analysis The mean differences with $\pm$ standard deviation (SD) were analyzed. Statistical analysis was performed using one-way analysis of variance test (ANOVA) ($p < 0.05$) followed by Tukey's test using the Statistical Package for the Social Science software (SPSS 22.0, SPSS Inc., Chicago, IL, USA). Data run for statistical analysis was triplicate observations.

3. Results and discussion

3.1. Particle size distribution

Protein particle size might be affected by the physical modification applications and particle size distribution depicts comprehensive information about heterogeneous solutions (Mirmoghtadaie et al., 2016). Size distributions of SPI and CAS as a function of intensity are presented in Table 1, and the graphs are shown in the supplementary file.

Native-SPI (N-SPI) dispersion showed three peaks and the mode of the dominant peak was 396.1 nm with 90.4% intensity. All MEF treatments decreased the size of the dominant peak and also increased the numbers of the smallest size particles (in a range between 8 and 22%) within the distribution. The v2 and V20 produced the SPI solutions with the smallest particle size. On the other hand, higher voltages applied for short times produced more homogenous SPI mass with smaller particle sizes. Lower voltage applications produced more heterogeneous solutions. The protein systems v2, V5 and V20 seemed to have some large diameter particles (> 4000 nm). This could be attributed to aggregation or partial opening of the globular structure of SPI with the effect of MEF applications (Li et al., 2007). The size and intensity of the big aggregates (V5) decrease when extending the application time (V20). Lower voltage applications were less prone to produce bigger aggregates than higher voltage applications. Prolonged application time is correlated with induced aggregation for protein particles (Jambrak, Mason, Lelas, Paniwnyk, & Herceg, 2014). This is correlated with the results of v1 and v2 applications.

Variance for the average hydrodynamic radius of SPI samples before and after treatments were also calculated (data not shown). All treatments reduced the hydrodynamic radius of dissolved SPI, compared with native form: 62% reduction with v1, 80% reduction with v2, 35% reduction with V5 and 58% reduction with V20 application was observed.

These results were consistent with particle size distributions. Relatively less reduction of the hydrodynamic radius obtained for V5 and V20 could be due to the aggregate formation with high voltages. The tendency for aggregation of SPI was possibly due to the crosslinking of bityrosine under an excessive external force like gamma irradiation, where the size of the aggregates increased at higher doses (Malik & Saini, 2017). The aggregates could be limiting for average particle hydrodynamic and diffusivities in the bulk solutions (Roebben et al., 2011). When considered the tendency of SPI particles for aggregation, filtration following the MEF treatment would decrease the aggregation potential during food processing.

Native-CAS (N-CAS) dispersion is characterized by three peaks with varying intensities (Table 1). The mode of the dominant peak was 342 nm with 77.2% intensity. It should be considered, N-CAS already had ~ 4000 nm big particles with ~ 20% intensity and a PdI value of 0.654. All applications except V20 produced smaller particles with higher intensities and more homogenous solutions compared to N-CAS. MEF effect on whey protein also provided smaller size particles with lower PdI values (Rodrigues, Fasolin, et al., 2020). 150 V exposure for 5 sec (V5) significantly produced the smallest particle solution with the highest intensity. In their study, Wang et al. (2019) emphasized that casein samples had smaller particles due to the shear force of ultra-high pressure application however, re-aggregation occurred when increased the applied pressure. Similarly, prolonging the application after 5 sec, the most intense aggregations were obtained within the next 15 sec (V20) throughout the solution. Consequently, 150 V MEF treatment had a drastic effect on CAS in terms of protein particle size.

Variance for an average hydrodynamic radius of CAS samples before and after treatments were calculated as 35% reduction with v1, 50% reduction with v2, 54% reduction with V5 while 5% increment with V20 (data not shown). V20 induced the protein particles to aggregate and increased the hydrodynamic radius correlated with the particle size distribution. This result was also confirmed with the MEF treated whey protein samples having the smallest hydrodynamic radius with the lower treatment periods (Pereira et al., 2016). When exposed to a shear force (electric field) and opposite reaction (drag force) the particle size of CAS decreased. Increased force broke down the casein polymers, destroyed the aggregates, however these thermodynamically unstable smaller-sized particles were prone to re-aggregation (Chunyang Wang et al., 2019). To prevent the re-aggregation and obtain homogenous protein solutions with smaller size, application parameters like treatment time or voltage should be considered.

Table 1

Polydispersity index (PdI) and the particle size distribution of SPI and CAS samples represented by the mode values (nm) and intensities (%) of the peaks in the bulk. 1, 2, 3 represent the number and order of the obtained peaks.

	Native			v1			v2			V5			V20		
SPI															
Peaks	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Mode (nm)	21	58.8	396.1	21	342		18.2	220	5560	23.2	295.3	5560	21	220.2	4801
Intensity (%)	2.2	6.3	90.4	10.2	89.9		23	72.1	4.2	24.3	52.3	23.4	17	71.4	11.7
PdI	0.385 ^c			0.687 ^b			0.752 ^c			0.399 ^c			0.302 ^d		
CAS															
Peaks	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Mode (nm)	50.7	342	4145	295.3	4801		225	5560		220.2	5560		7.5	531.2	5560
Intensity (%)	3.4	77.2	19.4	89.2	10.8		96.5	3.5		97.1	2.9		1	93.4	5.6
PdI	0.654 ^a			0.455 ^c			0.434 ^d			0.487 ^c			0.569 ^b		

*Different letters in the same row indicate significant differences ($p < 0.05$).

3.2. Interfacial Tension

In Table 2, the interfacial tension (IT) of native and treated SPI and CAS at 25 °C as a function of protein concentration were provided. Dependent on the structural alteration, hydrophobic groups that buried within the protein structure would reveal and might be affected on to decrease the interfacial tension (Dickinson, 2010). Measuring the IT of the samples was considered to obtain information about the MEF effect on the surface proteins at the air/water interface. On the other hand, the protein concentration is another important variable affecting the IT of the solution until the critical surface concentration (Razumovsky & Damodaran, 1999). The IT was observed as a function of concentration to see any trend alteration and identify the application which produces the lowest IT at the lowest protein concentration.

For SPI, all four applications significantly decreased the IT of water more than SPI-N at the concentrations 0.2% and 1% w/w. Interfacial tension of higher protein concentration might be lower compared with the lower concentrations due to the undesired excessive protein-protein interactions (Tomczyńska-Mleko et al., 2018). When considered the trends of all samples, V5 had similar behavior with SPI-N at every concentration except 0.2% w/w. On the other hand, V20 trend provided the lowest interfacial tension for all concentrations. SPI samples had smaller particles yielding better air/water interfacial activity as well as increased hydrophobicity after ultrasonic exposure (Malik, Sharma, & Saini, 2017b). These observations were also confirmed by Singh, Orsat, & Raghavan (2013) due to the increased surface hydrophobicity coupled with higher surface area of soybean proteins after moderate electric field processing. For the concentrations higher than 1% w/w, v1 showed a very similar trend with V20. Apparently, this is due to the concentration of 21 nm particles at the air/water interface. Since v2 had high amount of 18 nm particles, the IT was decreased up to 1% w/w concentration. Higher than 1% w/w, IT increased with increased v2 concentration. Due to the fact that 1% w/w could be considered as the critical concentration for v2 treated SPI. In order to detect the critical concentrations for other samples, IT as a function of broader concentration scale should be studied which is not in the scope of present study.

The lowest IT values of V20 at all concentrations were correlated with its highest homogeneity and having the smallest sized particles. However, this relation might not be applicable for every other protein solution samples and correlating the IT with PDI value may not be always straight-forward. Large protein aggregates in the protein solution with high PDI value prone to suspend or sink to the bottom and they cannot contribute to the air/water interfacial activity as much as dissolved unfolded protein particles on the surface. If a high

PdI protein solution includes dissolved surface protein particles coupled with suspended or sink forms, a lower IT value might be obtained under non-dynamic measurement conditions. For instance, the v2 produced the lowest IT at 1% (w/w) similar with V20 1% (w/w) however, it had the highest PdI among all samples.

For CAS, all four application had a lower trend than CAS-N however, IT value differences were statistically significant for only 0.2, 0.5 and 1.0% w/w concentrations. A behavior, looks like the critical concentration value for v1 was also observed at 1% w/w. At higher concentrations (1.5 and 2.0% w/w) all CAS samples had similar IT at the air/water interface. Since the CAS are structurally micellar associations, they are held together mostly with ionic or hydrophobic interactions (O'Regan, Ennis, & Mulvihill, 2009). MEF treatment would likely re-oriented these hydrophobic interactions that higher surface activity at the air/water interface was observed up to 1% w/w. At higher concentrations, micellar interactions possibly increased and no more significant difference was observed for any application. However, MEF applications affected and rearranged the CAS for particle size and air/water interfacial activity more than ultrasound treatment (de Figueiredo Furtado, Mantovani, Consoli, Hubinger, & da Cunha, 2017). Wang et al. (2019) confirmed the relation of smaller particle size whey protein for having better air/water interfacial activity after high-pressure treatment. Beside these static environment results, more detailed information about the non-thermal effect of MEF on proteins surface activity could be observed with a dynamic study (Benjamins, De Feijter, Evans, Graham, & Phillips, 1975).

Consequently, MEF treatments increased the surface activity of the dissolved SPI and CAS samples at the air/water interface, compared with their untreated forms. Higher interfacial activity that obtained at relatively lower protein concentrations for both samples was also consistent with the literature (Tomczyńska-Mleko et al., 2018).

Table 2

Interfacial tension (mN/m) of native and MEF treated SPI and CAS samples as a function of concentration at the air/water interface.

Sample	Protein Concentration (% w/w)				
	0.2	0.5	1	1.5	2
N-SPI	59.68 ± 0.9 ^a	55.95 ± 0.7 ^b	55.18 ± 0.1 ^a	53.17 ± 0.1 ^c	51.12 ± 0.2 ^c
v1-SPI	57.39 ± 0.1 ^c	57.63 ± 0.2 ^a	52.62 ± 0.7 ^c	50.85 ± 0.2 ^d	50.79 ± 0.2 ^{cd}
v2-SPI	58.41 ± 0.5 ^b	56.53 ± 0.1 ^b	51.21 ± 0.5 ^d	54.58 ± 0.7 ^a	53.88 ± 0.3 ^a
V5-SPI	54.41 ± 0.1 ^d	57.73 ± 0.3 ^a	53.94 ± 0.1 ^b	53.72 ± 0.1 ^b	52.00 ± 0.1 ^b
V20-SPI	54.46 ± 0.1 ^d	53.94 ± 0.5 ^c	51.70 ± 0.8 ^d	50.80 ± 0.1 ^d	50.52 ± 0.1 ^d
N-CAS	54.27 ± 0.1 ^a	51.98 ± 0.1 ^a	51.43 ± 0.1 ^a	51.16 ± 0.1 ^a	50.60 ± 0.1 ^a
v1-CAS	52.42 ± 0.1 ^b	51.35 ± 0.2 ^b	48.29 ± 0.1 ^c	50.15 ± 0.1 ^b	50.08 ± 0.1 ^b
v2-CAS	51.47 ± 0.1 ^c	51.14 ± 0.1 ^{bc}	50.75 ± 0.1 ^b	50.22 ± 0.1 ^b	50.46 ± 0.2 ^{ab}
V5-CAS	51.30 ± 0.2 ^c	51.48 ± 0.1 ^b	50.80 ± 0.1 ^b	50.66 ± 0.1 ^b	50.21 ± 0.1 ^{ab}
V20-CAS	51.65 ± 0.1 ^c	50.72 ± 0.1 ^c	50.70 ± 0.1 ^b	50.22 ± 0.1 ^b	50.30 ± 0.1 ^{ab}

Values are mean ± SD. Different superscripts in the same column for the same protein (for SPI or CAS, individually) indicate significant differences ($p < 0.05$).

3.3. Fourier Transform Infrared Spectroscopy (FTIR) spectra

FTIR spectra of SPI and CAS were presented in Fig. 2-A and 2-B, respectively. Three amide absorption bands for N-SPI (Fig. 2-A) were observed at 1632, 1515, and 1231 cm^{-1} , and were typically characteristic of C=O stretching (amide I), N-H bending (amide II), and N-H in plane and C-N stretching vibration (amide III), respectively. Amide I stretching and amide II shifts interpret the conformational analysis of protein's secondary structure (Das Purkayastha et al., 2016; J. Jiang, Zhang, Zhao, & Liu, 2018). The spectra did not change for amide I band, however, intensity changes were observed at 1632 cm^{-1} with MEF treatments. Only V5 sample had a higher intensity than N-SPI while the lowest intensity was observed for V20. The decrease of the intensity indicates a decrease for β -sheet content and an increase for α -helix content of the treated protein samples for SPI (Malik et al., 2017a). This was also confirmed with the amide II peak shift: The amide II peak shifted from 1515 (SPI-N) to 1521, 1522, 1520 and 1530 cm^{-1} in the spectrum of v1, v2, V5 and V20 respectively. It means the absorption peak moved to the longer wavelength, indicating that the SPI molecules had a

bulkier structure than N-SPI, and less energy for bending was required. The maximum and the minimum changes in this shift belong to V20 and V5, respectively. Protein's secondary structure due to unfolding is correlated with the decreasing the surface tension of water (Tomczyńska-Mleko et al., 2018). When considered the IT behavior trend of SPI samples, the lowest IT values obtained for V20, which had the maximum amide II peak shift and the highest values obtained for v5, which had the minimum amide II peak shift.

The changes of amide I and amide II bands also corresponded with the changes of α -helix and β -sheet contents indicating the protein structure loosening (J. Jiang et al., 2018). Researchers observed a reverse situation in high intensity ultrasound and gamma irradiation SPI treatment study. After treatment, α -helix content of the SPI decreased, β -sheet content increased and protein structure gained a better ordered form induced by protein-protein crosslinking (Malik & Saini, 2018; Malik et al., 2017a). Change in secondary structures, especially β -sheets is known as the indicator of protein denaturation (Wang, Li, Jiang, Qi, & Zhou, 2014).

The FTIR spectra of N-CAS and MEF treated samples showed no significant difference between the samples. This indicated no new generation of covalent bonds, as expected (J. Jiang et al., 2018). For CAS samples, the Fourier-deconvolution for amide II band was applied to see the changes of protein secondary structure (Fig. 2-B). The peaks around 1624, 1629 and 1638 cm^{-1} corresponded to β -sheet for N-CAS. 1642 and 1655 cm^{-1} peaks indicated some unordered formations and α -helix structures, respectively. The peaks representing the β -sheet content were shifted back with MEF treatment. Moving the absorption peak to the shorter wavelength indicates the treated CAS molecules had a finer structure than N-CAS, the more energy for bending was required and the β -sheet content was increased. Similar results were obtained for whey protein under moderate electric field, the increase of β -sheet content while decrease for α -helix alignment (Pereira et al., 2017, 2016). These results are also in correlation with smaller particle size distribution and the higher surface activities of MEF treated samples compared with N-CAS. The content of randomly coiled structures of CAS molecules decreased with MEF treatment. Moreira et al. (2019) indicated in their study about the ohmic heating effect on CAS, random coil structure might be decreased with MEF treatment and structure was rearranged. Based on the intensity changes around 1655 cm^{-1} , α -helix content of CAS samples decreased with MEF treatments. The max and the min decrease were obtained with V5 and V20, respectively. When considered the particle size distribution, the smallest size was obtained with V5 apparently with higher β -sheet content and the biggest size association obtained with V20 apparently

with the highest α -helix structures. The lower α -helix content corresponds the higher surface hydrophobicity of for the protein (Wang et al., 2011). Higher activity at the air/water interface also confirmed this observation for MEF treated samples.

Consequently, the MEF treatments V5 and V20 mostly affected the secondary proteins structures for both SPI and CAS, however in the opposite ways. The reason for this difference is most likely the natures of the proteins; SPI, a globular 11S dominated protein and CAS, a micellar protein constituted mostly secondary structures holding together ionic/hydrophobic forces. These results are consistent with other studies in the literature; higher/increased α -helix content of globular soybean and pea protein (with pH alteration) resulted with increased surface activity (Jarpa-Parra et al., 2015; Wang et al., 2011). On the other hand, decreased α -helix content for casein (with electric field treatment) and for whey protein isolates (with phenolic interaction) were also reported (J. Jiang et al. 2018; Pereira et al., 2010). The secondary structure changes in these studies were all interpreted as the unfolding of the proteins.

Fig. 2. FTIR spectra of SPI (A) and Fourier-deconvoluted FTIR spectra of CAS (B).

3.4. Intrinsic Fluorescence Spectroscopy

Intrinsic fluorescence spectroscopy provides information about the tertiary structure of a protein (Vivian & Callis, 2001). Emission spectrum belonging to SPI samples is presented in Fig. 3-A. The recorded emission is dominated by tryptophan among all, due to its higher absorptivity and quantum yield (Helo, 2006). When compared with N-SPI, all treatments produced lower intensity of tryptophan emission. This indicates that tryptophan moieties were less exposed to excitation and lower emission intensities were detected. Fluorescence property of tryptophan is dependent on the polarity of its microenvironment. Tryptophan residues assigned to be buried in hydrophobic, nonpolar zones of tertiary structure. When protein structure unfolds, tryptophan residues contact with aqueous polar media and fluorescence signals of tryptophan quench (Vivian & Callis, 2001; Lakowicz, 2006). On the other hand, the emission maxima of all treated samples were shifted from 324 to 326 nm for all SPI treated samples. This red shift in the emission maxima also confirmed the increased contact of fluorophore with an increased environment polarity (J. Jiang et al., 2018). Consequently, tertiary structures of the protein isolates treated by all four applications were different than that of N-SPI. Fluorescence quenching of tryptophan could also be attributed to the conformational changes of the tertiary structure, breaking the hydrophobic bonds, aggregations and/or crosslinking of the side chains of amino acids with an outer driving force: modification applications (Chen & Barkley, 1998; Chen, Liu, Yu, & Barkley, 1996). With

lower particle size distribution and higher air/water interfacial activity of MEF treated SPI samples were the confirmations of the tertiary structure alteration.

Fig. 3-B. represents the emission spectrum of CAS samples before and after MEF treatments. All treated CAS samples produced higher fluorescence intensities than N-CAS. However, only the emission maxima of v2 and V5 was shifted from 328 to 330 nm while no shifting was observed for v1 and V20. Red shift of emission maxima indicates the changes of tertiary structure as mentioned before. Shifting of emission maxima is dependent on the location of tryptophan within the protein molecule. If tryptophan is buried in a hydrophobic core, then the emission maxima would shift. If tryptophan is located as in contact with the environment (not buried in a hydrophobic core) or close to the surface, no shifting might be observed for emission maxima (Burstein, Vedenkina, & Ilyukova, 1973). Unfolded and/or partially denatured protein molecules under electrical strength exposed to drag force in the same direction of the electrical field. Hydrodynamic friction between the water and protein molecules promotes hydrophobic interactions to break down while changing particle size, surface area and creates a novel surface (Jambra et al., 2014). Treated and partially unfolded protein molecules rearranges them to gain a lower energy state (Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011; Güçseren, Güzey, Bruce, & Weiss, 2007). New hydrophobic/ionic interactions aid to build smaller protein pockets. Yu et al. (2014) demonstrated that aromatic ring carrier proteins buried inside the globules of α -amylase had a novel conformation with the effect of sonication dependent high shear. Embedding of aromatic amino acids into novel protein pockets with conformational rearrangements explains the increment of fluorescence intensity. With a red shift of emission maxima and yielding the highest intensity, the V5 altered the CAS conformation more than other treatment, which is also confirmed with the secondary structure results as well as having the smallest particle size distribution. Abdualrahman et al. (2016) indicated that ultrasound pretreated CAS samples produced red shift in their spectra from 350 to 354 nm and increased fluorescence intensity, similar to our results. Briefly, MEF treatments changed the tertiary structures and hydrophobic interactions of SPI and CAS in different ways compared with their native forms.

Fig. 3. Intrinsic fluorescence spectra of native (N) and MEF treated SPI (A) and CAS (B)

3.5. Differential scanning calorimetry (DSC)

Differential scanning calorimeter (DSC) is a tool that helps to clarify protein denaturation, aggregation, folding or thermal stability phenomena (Johnson, 2013). Glass transition temperature (T_g), onset temperature (T_{onset}), peak temperature (T_{peak}) and total enthalpy change (ΔH) are the most important thermal indicators provided by DSC (Parniakov et al., 2018). These thermal properties of native and MEF treated SPI and CAS samples are provided in Table 3.

High T_{peak} value is correlated with high thermal stability; furthermore, T_{peak} value provides information about protein tertiary structure: the more compact the tertiary

conformation, the higher T_{peak} value is obtained (Tang & Sun, 2011). There is a direct relation between the size of the particles while the relation is inverse with the PdI value: the bigger the particle size and a lower PdI, the T_{peak} is higher (Shiba et al., 2010). The ΔH expresses the energy required to unfold protein's tertiary structure. It is related with the secondary structure of the protein system (Meng & Ma, 2001).

Denaturation temperature (T_{peak}) and denaturation energy (ΔH) of all treated SPI samples were lower than that of N-SPI, as expected from the smaller particle sizes, secondary structure and tertiary conformational changes. Following N-SPI, the highest T_{peak} was observed for V5 and the lowest for the V20, similar with their structural changes. Denaturation enthalpy of all treated samples were quite lower than N-SPI. Lower ΔH value is also another important indicator for protein structure differentiation. After treating SPI with ultrasound and gamma irradiation, T_{peak} and ΔH values increased or decreased together in parallel and followed a trend depending on the treatment parameters as a function of conformational change (Malik, Sharma & Saini, 2017a, 2017b).

Denaturation temperature (T_{peak}) of CAS samples decreased with all MEF treatments and the lowest T_{peak} was obtained for V20. The V20 was seemed to disrupt the hydrogen bonds that may exists during aggregation, more than other applications (Tang & Sun, 2011). This was expected due to the secondary structure changes because the V20 exhibited the least β -sheet formation. On the other hand, denaturation energy (ΔH) of N-CAS was the highest as 5.17 J g^{-1} and it was followed by V5. The V5 had the highest β -sheet formation among all treated samples. However, as being a micellar association, ΔH was affected from the particle size distribution more than structural conformation. The v1 was the only sample not having aggregates over 5000 nm and having a lower PdI at the same time (Shiba et al., 2010).

Table 3. Thermal properties of native and MEF treated SPI and CAS

Sample	T _g (°C)	T _{onset} (°C)	T _{peak} (°C)	DH (J g ⁻¹)
N-SPI	30.02 ± 0.9 ^c	95.79 ± 0.9 ^a	116.83 ± 1.2 ^a	2.03 ± 0.2 ^a
v1-SPI	23.82 ± 0.4 ^d	90.85 ± 0.4 ^c	97.64 ± 0.9 ^c	0.44 ± 0.1 ^c
v2-SPI	-----	84.31 ± 0.7 ^e	97.63 ± 1.1 ^c	1.08 ± 0.1 ^b
V5-SPI	39.01 ± 0.1 ^b	94.50 ± 0.7 ^b	105.61 ± 1.4 ^b	0.24 ± 0.1 ^d
V20-SPI	41.04 ± 0.6 ^a	87.70 ± 0.3 ^d	96.60 ± 1.0 ^d	0.38 ± 0.1 ^{cd}
N-CAS	48.20 ± 0.6 ^a	84.15 ± 0.6 ^a	103.71 ± 0.8 ^a	5.17 ± 0.6 ^a
v1-CAS	40.50 ± 0.3 ^c	80.36 ± 0.6 ^c	102.09 ± 0.5 ^b	3.66 ± 0.2 ^d
v2-CAS	41.04 ± 0.8 ^b	80.34 ± 0.8 ^c	100.34 ± 0.6 ^d	4.47 ± 0.3 ^b
V5-CAS	38.88 ± 0.5 ^d	75.42 ± 0.5 ^d	100.93 ± 0.6 ^c	4.57 ± 0.3 ^b
V20-CAS	40.43 ± 0.2 ^c	82.23 ± 0.9 ^b	96.85 ± 0.4 ^e	4.32 ± 0.2 ^c

Values are mean ± SD. Different superscripts in an individual column for the same protein (for SPI or CAS, individually) indicate significant difference (p < 0.05).

4. Conclusion

MEF affected the protein particle size and homogeneity, interfacial tension at the air/water interface, secondary and tertiary structures and thermal properties of both SPI and CAS. All treatments produced lower particle size SPI solutions with varying homogeneities, however, the solution with the smallest particle size and the highest homogeneity was obtained with V20. Similarly, the lowest stable surface tension for all measured protein concentrations was produced with V20. SPI protein's secondary and tertiary structure changed mostly with V20 and gained the most bulkier, flexible structure and surface hydrophilicity. The lowest denaturation temperature was obtained also with V20 but the V5 had minimum denaturation enthalpy. Heat stable, 11S helianthinin dominated globular SPI was seemed structurally changed mostly with short time applications under 150 V electric field.

On the other hand, CAS was effected mostly with lower voltage (10 V) treatments for both v1 and v2, in terms of having the smallest particle size with the most homogenous distribution as well as the lowest surface tensions for all prepared concentrations. The v1 also produced the CAS solution that has the lowest denaturation enthalpy. Secondary structure of the CAS mostly effected from V20. The most hydrophobic structure was also obtained with V20 as well as increased particle size, possibly due to the aggregation of micelles. Breaking down the ionic/hydrophobic interactions with the effect of electrical friction possibly

produced novel protein pockets with renovated interactions for all MEF treatments. Consequently, the non-thermal effect of MEF treatments could be proposed as a promising physical method to change the protein's structure as well as particle size and thermal properties. Structural changes like unfolding or partial denaturation of a protein considered the keys for better functional properties, therefore further investigations are required.

Declaration of competing interest

The authors declare no conflict of interest.

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Author Statement

Büşra Gültekin Subaşı and Mastaneh Jahromi: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Writing - Original Draft, Writing - Review & Editing. **Federico Casanova and Fatemeh Ajalloueian:** Conceptualization, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization. **Esra Capanoglu and Mohammad Amin Mohammadifar:** Writing - Review & Editing, Visualization, Supervision, Project administration and Funding acquisition.

Journal Pre-proof

Highlights

- Non-thermal effect of moderate electric field on protein structure was investigated.
- Treated SPI and CAS present lower air/water surface activity and higher hydrophilicity.
- Smaller sized particle distributions were observed for treated proteins.
- Lower denaturation temperature and enthalpies were observed for treated protein.

Figure 1

Figure 2

Figure 3