Effect of Pulsed Light Treatment on Structural and Functional Properties of Whey Protein Isolates

Md Abu Bakar Siddique[a], Paola Maresca[b], Gianpiero Pataro[a*], Giovanna Ferrari [a,b]

[a]*Department of Industrial Engineering, University of Salerno,84084 Fisciano (SA), Italy*

[b]*ProdAl S.c.ar.l.,84084 Fisciano (SA), Italy*

*Corresponding author: Tel.: +39089969439; Fax: +39089964168
*E-mail address:*gpataro@unisa.it *(G. Pataro)*

Abstract

This work aimed at investigating the effects of Pulsed Light (PL) processing at different energy doses (from 4 to 16 J/cm^2) on the structure and functional properties of Whey Protein Isolate (WPI) solutions. The determination of the free and total sulfhydryl (SH) groups was used to detect the variation of WPI tertiary and quaternary structure. Additionally, PL-induced changes in secondary structure were determined by FT-IR spectroscopy and the differential scanning calorimetry (DSC), and primary structure by carbonyl content.

The experimental data demonstrated that PL treatments increased the concentration of total and free sulfhydryl groups and protein carbonyls. A decrease of the denaturation temperature and enthalpy ratio with increasing the intensity of PL treatments was observed in DSC measurements. Small but significant changes in the secondary structure of PL treated WPI solutions were also taking place and detected. The extent of whey protein structure modifications was energy dose dependent. The results of this investigation demonstrated the potential of PL treatments to induce dissociation and partial unfolding of WPI, thus improving some of their functional properties, such as solubility and foaming ability.

Keywords: *Pulsed light, whey protein, unfolding, solubility, foaming properties, conformational structure.*

1. Introduction

Proteins are the main structural and functional components of many food systems, e.g., meat, cheese, gelatine, egg white and most of the cereals. In addition, proteins are being increasingly used to facilitate the engineering and fabricate new food products, such as protein beverages and extruded foods. These applications depend upon the physicochemical properties of protein ingredients, collectively referred to as their functional properties (Kinsella, 1982). The effectiveness of the use of proteins in food processing depends on their functional characteristics, which can be tailored to meet the complex needs of food products manufacturers (Chobert, 2003). Among other components, dried whey powders, a major by-product of cheese and casein manufacturing, have become a well-known ingredient of the food industry due to the low price, versatile functionality, and high nutritional value (Kim et al., 1981).

Whey proteins (WP) are a mixture of β-lactoglobulin, α-lactalbumin, Bovine serum albumin, Immunoglobulins, proteose-peptones, and other minor proteins. In their native form they exist as compact, globular proteins (Lee et al., 1992). Although the normal functionality of WP mainly depends on the behaviour of β-lactoglobulin, the most abundant protein in whey (Verheul et al., 1998), the overall functionality depends on the combined properties of all WP components.

In general, several factors affect the functional properties of food proteins, namely intrinsic factors such as amino acid sequence and composition, secondary and tertiary structures, hydrophilic/hydrophobic character of the protein surface, net charge and charge distribution and molecular rigidity/flexibility of the protein and extrinsic factors such as pH, ionic strength, temperature and interactions with other food components (Zhu & Damodaran, 1994).

Food processing can affect the functionality of proteins since it may modify the native structure of the protein reversibly (unfolding) or irreversibly (denaturation) depending on the technology used and the processing conditions applied. Food, chemical and pharmaceutical industries, which utilize WP as technological ingredients in their productions, are interested to control proteins functional properties with the aim of improving the stability of the formulations or developing novel foods (McClements, 1999). To this purpose, in the current literature several papers have focused on the application of different treatments based on chemical, physical, or enzymatic methods, to induce the desired structural modifications of proteins, thus improving their functional properties (Davis, Doucet, & Foegeding, 2005; Dissanayake & Vasiljevic, 2009; Enomoto et al., 2007; Nicorescu et al., 2009).

Recently, researchers of the R&D departments of the food industry and of research institutions proposed the utilization of non-thermal processing technologies to selectively modify the structural and functional properties of proteins in order to obtain protein derivatives with targeted functionality appropriate for different formulated foods (Dissanayake, Ramchandran, Piyadasa, & Vasiljevic, 2013). High pressure processing, ultrasounds, pulsed electric fields, UV light and ozone have been used to modify milk proteins conformational structure (Bouaouina, Desrumaux, Loisel, & Legrand, 2006; Chandrapala, Zisu, Palmer, Kentish, & Ashokkumar, 2011; Kristo, Hazizaj, & Corredig, 2012; Segat et al., 2014, Xiang, Ngadi, Ochoa-Martinez, & Simpson, 2009).

Among non-thermal technologies, Pulsed Light (PL) has drawn considerable attention as gentle but targeted processing technique, which can be used, as an alternative to chemical and thermal methods, to decontaminate foods and food contact surfaces with minimal losses of nutrients and flavour (Oms-Oliu, Martin-Belloso & Soliva-Fortuny, 2010). PL process consists of the exposure of food product to a series of short (100 ns-1ms), high-intensity pulses (flashes) of

77 polychromatic light (200 nm – 1100 nm) including UV (180-400 nm), visible (400-700 nm) and
78 near infrared region (700-1100 nm) produced by a xenon flash lamp (Wekhof, 2000). Although
79 this technology has been successful applied to inactivate a number of pathogenic and spoilage
80 microorganisms in vitro or in food products (Oms-Oliu et al., 2010), only very few data have
81 been published discussing the effects of PL on proteins structure. Fernández et al. (2012)
82 reported that PL treatments can improve surface and foaming properties of β-lactoglobulin, while
83 Elmnasser et al. (2008) described the effects of PL treatment on milk proteins and lipids
84 observing minor changes in proteins structure. However, the impact of PL treatments on the
85 structure as well as on the functional properties of WP has not been investigated yet.
86 This work aimed to investigate the effects of PL treatments on some key functional properties of
87 Whey Protein Isolate (WPI) and understand and elucidate the mechanism of interaction between
88 PL and WPI structure. Different PL energy doses (fluences) were utilized to induce WPI
89 unfolding. The proteins unfolding was assessed determining the total and free sulfhydryl (SH)
90 groups, solubility and the foaming ability. The changes of the secondary structure were
91 determined by FT-IR spectroscopy and differential scanning calorimetry (DSC), while the effect
92 on primary structure was assessed by the determination of the carbonyl content.

2. Materials and Methods

2.1 Materials and sample preparation

Powder of WPI (UltraWhey 90 instant) derived from sweet cheese whey was purchased from Volac International Ltd. (Orwell, UK) and stored at 4 °C until used. The weight composition of the powder was as follows: 90% proteins, 1.0% fat, 2.0 % lactose, 2.2% ash, and 4.0% moisture. The protein fraction included β-lactoglobulin (43-48%), α-lactalbumin (14-18%), Bovine serum albumin (1-2%), Immunoglobulin G (1-3%), and Lactoferrin (<1%).

All chemicals used in this study were from Sigma Aldrich (Milan, Italy) unless otherwise stated. MilliQ water was used to dilute samples and prepare all reagents and buffers.

2.2 Sample preparation

Before undergoing PL treatments, WPI powder was dissolved in Sodium phosphate buffer (50 mM; pH=7.5) and maintained under gentle mixing in a water-ice bath in order to obtain a homogenous solution. The WPI solution with a final concentration of 1% (w/v) was then stored at 4 ^{o}C until use. The pH of the protein solution was measured with a pH-meter (S400 Seven Excellence, Mettler Toledo International Inc.).

2.3 PL treatments

PL treatments were performed in a bench-top RS-3000C SteriPulse-XL system (Xenon Corp., Wilmington, Mass., USA), described in details in Pataro, Sinik, Capitoli, Donsì, & Ferrari (2015). In brief, the system included a power/control module, a rectangular parallelepiped treatment chamber made in stainless steel, and a lamp housing placed on the top of the chamber and containing a linear 16” xenon flash lamp and a forced air cooling system. A quartz window, placed at 5.8 cm from the lamp source, was used to separate the lamp housing from the treatment chamber. The system generated high intensity pulses of polychromatic light in the wavelength range between 200 and 1100 nm at a pulse rate of 3 pulses/s and 360 μs duration. An adjustable stainless steel tray in the treatment chamber allowed changing the vertical distance between the sample and the quartz window surface from 1.9 to 16.5 cm.

Before PL treatment, 2 mL of the refrigerated (~4 °C) WPI solution were poured in a Petri dish (3.5 cm in diameter) to cover the entire dish surface to a depth of 2 mm. Afterwards, the Petri

dish was placed in the centre of the tray into another Petri dish (9.5 cm in diameter) containing ice flakes which allowed to minimize the temperature increase of the sample during the light treatment. The experiments were carried out by placing the samples at a vertical distance of 10.62 cm from the quartz window, at which correspond an energy dose per pulse of 0.43 J/cm^2, for an input voltage of 3800 V, as per manufacturer specification. Samples were exposed to irradiation with PL for 3.1, 6.2, 9.3 and 12.4 s, corresponding to total energy doses (fluences) applied of 4, 8, 12 and 16 J/cm^2, respectively.

After treatment, samples were collected in plastic tubes wrapped in aluminium foil. Untreated samples were also taken and used as control. The samples were, then, immediately placed in ice until analyses.

In all experiments, the initial temperature of the samples was 4 ± 2°C. The temperature increase of the samples exposed to PL treatments was monitored utilizing a 0.5 mm K-type thermocouple submerged into the treated solution. With increasing the energy dose at 4, 8, 12, and 16 J/cm^2, the temperature of the samples increased gradually of 10.2, 12.3, 14.2, and 14.7 °C, respectively.

2.4. Samples analyses

The analytical protocols utilized to detect the changes of the structural and functional properties induced by PL irradiation in WPI samples are reported in the following.

2.4.2 Determination of free SH-groups

The concentration of total and free sulfhydryl (SH) groups in the untreated and PL treated WPI solutions, was determined by the modified Ellman's assay method (Ellman, 1959), previously described by Kehoe, Remondett, Subirade, Morris, & Brodkorb (2008), with some slight modifications. For each assay, WPI solution was diluted up to a concentration of 2 g/L protein in a 50 mM Tris-HCl buffer (pH 7.0) for the determination of free SH-groups or in a 50 mM Tris-HCl (pH 7.0) with the addition of 8 M urea for the determination of total SH-groups. Then, 2.75 mL of each diluted solution were placed into a centrifuge tube and added to 0.25 mL of a 1 g/L DTNB solution in 50 mM Tris-HCl buffer. The solutions were incubated in the dark at room temperature. After 30 min, absorbance was measured at 412 nm by a UV/VIS spectrophotometer (V-650, Jasco Europe Srl, Italy), and the concentration of sulfhydryl groups (in μmol SH/g WPI)

was calculated according to the formulae reported by Segat et al. (2014) using a molar absorptivity constant equal to 1.36×10^4 $M^{-1}cm^{-1}$.

2.4.3 FT-IR measurements

Attenuated total reflection infrared (ATR FT -IR) spectra of untreated and PL treated WPI solutions were measured according to the protocol described by Yang, Kong, Dong, & Yu, (2015) utilizing a Jasco FTIR-4100 series spectrophotometer (Maryland, United States) equipped with a highly sensitive and stable DLATGS detector. A specially designed vibration-proof mounting of the optical bench eliminates completely the interferences due to external vibrations. The spectra were measured at the maximum resolution of the instrument (0.9 cm^{-1}) in the wavenumber range between 650 and 4000 cm^{-1} in the double-sided, forward-backward mode. Background spectra were collected and used to remove spectral signals that originated from air, moisture (water vapour) and coating materials on reflecting mirrors along the IR radiation path from the spectra of protein and buffer in order to subtract the background noise. Data analysis was initiated by collecting IR absorbance spectra and subtracting reference from the protein spectra to remove water contribution using a double-subtraction procedure. Baseline correction was done as a pre-processing step to account for and correct noise and sloping baseline effects. The region of interest for Amide I was selected from 1700-1600 cm^{-1}. Band narrowing of the spectra was done by performing second-derivative analysis. The spectra were processed by Origin Pro 8 software (OriginLab Corporation, Northampton, USA).

2.4.4 Differential scanning calorimetry (DSC) analysis

Thermal analysis of untreated and PL treated WPI samples was carried out using a differential scanning calorimeter (NETZSCH DSC 204 *F1 Phoenix, US*) equipped with NETZSCH Proteus Software. 20 µL of WPI solutions were weighed into aluminium pans and hermetically sealed. An empty pan of equal weight was used as reference. The scanning temperature was raised from 25 to 100 °C at a rate of 10 °C/min.

The onset (T_{onset}) and the offset (T_{offset}) temperatures are defined as the intersection of the tangents of the peak with the extrapolated baseline, and the peak denaturation temperature (T_d) is defined as the temperature at the maximum/minimum of the thermal event. These temperatures were read from the corresponding thermogram, while the denaturation enthalpy (ΔH) was

obtained from the integration of the thermogram. Denaturation enthalpy ratio (ΔH_{ratio}) was also calculated as the ratio between the enthalpy of PL treated and untreated WPI sample. The analyses were carried out in duplicate.

2.4.5 Measurement of protein carbonyls

Protein carbonyls content of untreated and PL treated WPI samples was determined according to the protocols reported by Levine et al. (1990) with some modifications. An aliquot of WPI solution (corresponding to 2 mg of protein) was incubated with 10 mM 2, 4-Dinitrophenylhydrazin (DNPH) in 2M HCl (1mL), for 30 min at room temperature (25 °C). A 10% (w/v) trichloroacetic acid (final concentration) was used to precipitate proteins, which were recovered by centrifugation at 6,500 × *g* for 5min (ALC PK130 refrigerated centrifuge, Cologno Monzese (MI), Italy). Protein pellets were washed three times with 1 mL of ethanol/ethyl acetate 50:50 (v/v) to remove residual unreacted molecules of DNPH and dissolved in 1 mL of 6M urea (pH=2.3). The concentration of protein carbonyls, were determined by an UV/VIS spectrophotometer at 370 nm using an extinction coefficient of $2.2 \times 10^4\ M^{-1}cm^{-1}$ (Scheidegger, Pecora, Radici, & Kivatinitz, 2010). The results were expressed as nmol of carbonyl/ mg of protein.

2.4.6 Protein Solubility

Untreated and PL-treated WPI solutions (10 g/L) were diluted to reach a final concentration of 2 g/L and centrifuged at 6000 rpm for 15 min at 4 ^{o}C. Soluble protein content in the supernatant was determined at room temperature (25 ^{o}C) by the modified method of Lowry, Rosebrough, Lewis, and Randall (1951), using Bovine serum albumin (BSA) (Sigma Chemical Co., Louis, MO) as standard. The percentage reduction of protein solubility was calculated according to Eq.1:

$$Solubility(\%) = \frac{\text{Protein in the supernatant}}{\text{total protein}} \text{x } 100 \quad (1)$$

The analyses were performed in duplicate.

2.4.7 Foaming Properties

Foaming capacity and stability of untreated and PL treated WPI solutions were determined as previously reported by Segat et al. (2014), with slight modifications. 10 mL of WPI solutions were homogenized at 12,000 rpm for 2 min using an IKA Ultra TurraxT25 digital homogenizer (IKA®-Werke GmbH & Co. KG) in graduated tubes. Foaming capacity (FC) and foam stability (FS) were calculated according to Eq.2 and Eq.3 as follows:

$$FC(\%) = \frac{V_{Fo} - V_L}{V_L} \text{x } 100 \quad (2)$$

$$FS(\%) = \frac{V_{F30}}{V_{Fo}} \text{x } 100 \quad (3)$$

where V_L, V_{F0}, and V_{F30} represent the volume of non-whipped WPI solution and the volumes of the foam immediately after whipping (time 0) or after standing at room temperature for 30 min, respectively.

2.5. Statistical analysis

Every PL treatment was performed twice and all analyses were repeated three times unless otherwise specified. The mean values and standard deviations (SD) of the experimental data were calculated. Differences among mean values were analysed by means of one-way ANOVA test in order to determine whether different values of PL energy dose led to a significant difference in the conformational and functional properties of the samples. When significant differences were detected, the Tukey test was performed to determine which particular means were significantly different *($p \leq 0.05$)*. The Pearson product-moment correlation coefficient was used to measure the strength of the linear relationship between two variables. Statistical analyses were carried out using IBM SPSS statistics 20 software.

3. Results and Discussion

3.1. Changes in the conformational structure of WPI

3.1.1 Determination of SH-groups

In WPI, β-lactoglobulin is known to have 1 free –SH group (Cys121) and 2 S–S bonds (Cys66–Cys160 and Cys106–Cys119) (Hambling, McAlpine, & Sawyer, 1992), while α-lactalbumin does not contain any free–SH groups but has 4 S–S bonds (Cys6–Cys120, Cys28–Csy111, Cys61–Cys77, and Cys73–Cys91) (Brew, 2003).

The analysis of the free and total SH-groups may be considered as an indication of protein unfolding and/or denaturation. In fact, it is known that disulphide bonds can stabilize the folded conformation and decrease the conformational entropy of proteins, thus improving their thermodynamic stability (Sun, 2010). Generally, SH groups in the native proteins are located in poorly accessible regions of the polypeptide chain, thus, are masked to the attack by Ellman's reagents used in the analytical assay. If an external stress is able to modify the conformational structure of a protein, an increased content of SH groups may be detected according to the protocol of the Ellman's reaction (Beveridge, Toma, & Nakai, 1974; Hardham, 1981).

The effect of PL treatments at different energy doses on the concentration of total and free SH-groups of untreated (0 J/cm^2) and PL treated samples, is shown in Fig. 1.

In our experiments WPI samples showed an initial content of free SH-groups of 0.6 μmol/g of protein, while the concentration of total SH-group was 3.7 μmol/g of protein (Fig. 1). The exposure to PL treatments at different energy doses (from 4 to 16 J/cm^2) significantly *($p \leq 0.05$)* increased the concentration of both total (from 4.5 to 6.2 μmol/g) and free SH-groups (from 1.6 to 3.9 μmol/g), as compared with the control sample.

The results obtained demonstrate that, similarly to thermal (Hoffmann & van Mil, 1999) and high hydrostatic pressure treatments (Qin et al., 2012), PL processing is able to induce protein unfolding, which allows the masked SH groups to be exposed and consequently detected.

Our experimental data are in agreement with those of Neves-Petersen et al. (2002) and Permyakov et al. (2003). Neves-Petersen et al. (2002) investigated the reaction mechanism behind the UV light-induced reactions on cutinase samples. The authors observed an electron ejection from the side chains of aromatic residues, namely tryptophan, upon UV illumination of aromatic amino acids, and the transfer from an excited tryptophan amino acid to a neighbouring disulphide bond. Because disulphide bridges are excellent quenchers of the excited state of aromatic residues, a cleavage of the disulphide bridge can be hypothesized, thus leading, consistently, to the detection of an increasing content of free thiol groups with increasing the energy dose. Similarly, Permyakov et al. (2003) also stated that UV-induced changes in the properties of α-lactalbumin resulted from the reduction of disulphide bonds as a consequence of transfer of energy from the nearby excited tryptophan chromophores.

Moreover, as reported by Hoffmann and van Mil (1999), reaction of DTNB with the thiol groups (SH-groups) in Ellman's protocols can be distinguished in two steps: (1) diffusion of DTNB into the thiol group and (2) reaction of DTNB with the thiolate anion. However, at neutral pH, thiol

group in native β-lactoglobulin is shielded by other groups, thus DTNB needs to diffuse into the molecule before the reaction can occur. Thus, considering the experimental data reported in Fig. 1, it is likely that PL treatments were able to modify the conformational structure of the treated WPI samples, enhancing the diffusion of DTNB and promoting its reaction with SH-groups.

Moreover, it should be also considered that the unmasked SH groups (dissociation and initiation step) are more reactive and could disappear because of sulfhydryl-disulphide bond exchange reactions, which are especially promoted at alkaline pH causing a decrease in free SH content due to the oxidation of the thiols and the formation of disulphide bonds.

In contrast, our results show that, within the range of energy doses investigated, after PL treatments an increasing concentration of SH-groups was detected. It has been previously stated that the placement of SH-groups in hydrophobic regions can occur either by folding of a single protein molecule or by the aggregation of subunits as into dimmers, tetramers etc. (Ustunol, 2015). Therefore, these results have suggested that PL induced the partial unfolding of WPI, leading to, consequently, the exposure of the internal SH-groups buried inside to the surface.

Further PL treatments, utilizing a range of energy doses wider than that investigated in this work, should be carried out in order to verify the existence of a threshold value of the energy dose, above which the content of SH-groups decreases, leading to protein aggregation. This has been already observed for other non-thermal technologies. For example, Qin et al. (2012) utilized high hydrostatic pressure to process protein, and detected a higher number of SH groups when the treatment was carried out in the pressure range between 300-400 MPa, while a significant decrease of SH groups was observed when the proteins were processed in the pressure range of 400-500 MPa. A similar trend was also observed by Li, Chen, & Mo (2007) when soybean protein isolates were exposed to pulsed electric fields treatments.

3.2 Effect of PL on the secondary structure of whey proteins

3.2.1 FT-IR analysis

PL induced changes of protein secondary structure was determined by FT-IR spectra measurements performed at room temperature. This technique, in fact, is known to provide highly accurate information on the secondary structure of proteins in water solutions as well as in deuterated forms and dried states (Byler & Susi, 1986). Differences among the spectra of the samples analysed were identified by band narrowing approach of the second derivative

spectroscopy. Fig. 2 shows the FT-IR spectra of untreated and PL treated WPI samples in the Amide I (1700 to 1600 cm^{-1}) region. Generally, protein structural units are able to absorb specific wavelengths of radiation in the infrared region, giving rise to a characteristic set of nine absorption bands, namely Amide A, B and Amide I-VII. Among them, Amide I and II represent the vibrational bands of the protein backbone and are sensitive to the secondary structure of a protein. In particular, the absorption associated with the Amide I band represents the stretching vibrations of the C=O bond (approximately 80%) of the amide groups, while, according to several studies reported in the literature, Amide II band is not as good a predictor for quantifying the secondary structure of proteins (Fabian & Mäntele, 2002; Krimm & Bandekar, 1986; Surewicz & Mantsch, 1987).

The spectrum of Amide I band typically consists of a band contour which is composed of several overlapping bands due to various protein segments with different secondary structure (Surewicz & Mantsch, 1987).

The second derivative of the FT-IR spectra in Amide I region, allowed to identify seven major peaks corresponding to α-helix (1648-1657 cm^{-1}), unordered (1642-1675 cm^{-1}), β-sheet (1620–1610 cm^{-1}), β-turn (1690–1670 cm^{-1}), and β-antiparallel sheet (~1690 and 1641–1623 cm^{-1}) according to the assignments by Barth (2007). Parris, Purcell, and Ptashkin (1991) also reported assignments of the seven bands in the Amide I region for β-lactoglobulin.

According to the experimental data plotted in Fig. 2, variations in the shape and area of bands were observed due to PL effect on the WPI samples. Amide I band of untreated whey protein exhibited an absorbance peak, which corresponded to a wavenumber of at 1651 cm^{-1} and could be attributed to α-helix. A slightly narrow peak at 1635 cm^{-1} could be associated with the formation of intra-molecular β-sheet. With increasing PL energy from 4 to 12 J/cm^2, the peak intensity at 1651 cm^{-1} increased at a higher rate in comparison to the peak intensity at 1636 cm^{-1}. A further increase of the energy dose up to 16 J/cm^2 caused the disappearance of the peak at 1658 cm^{-1} and the formation of a broad peak at 1663 cm^{-1}. Moreover, gradual shifting of the peak at 1675 cm^{-1} to lower wavenumber (1673 cm^{-1}) was also observed with increasing the energy doses. These changes clearly indicated that PL affected the secondary structure of WPI samples.

Furthermore, upon PL treatments, β-sheet structures (peak at 1616 and 1628 cm^{-1}) were replaced by an unordered (random coil) structure corresponding to the peak at 1644 cm^{-1}. Similarly, Zhu and Damodaran (1994) described a progressive decrease in β-sheets along with an increase in

unordered structures while studying heat-induced conformational changes in WPI with different heating times.

Results of Fig. 2 also show that another noticeable peak was formed at 1613 cm^{-1} for the samples processed at higher energy doses in the investigated range. This peak is mainly associated with intermolecular β-sheet structure resulting from aggregation before gelation (Byler & Purcell, 1989).

In conclusion, the findings deriving from the FT-IR analysis seem to confirm the assumption of partial unfolding of protein structure induced by PL treatments, which could promote the formation of aggregates.

3.2.2 DSC analysis

DSC characteristics have been extensively used to study the thermodynamic and kinetics of protein denaturation. Changes in thermodynamic properties of the proteins, such as denaturation temperature (T_d) and enthalpy (ΔH) during thermally induced protein denaturation can reflect the unfolding and denaturation of proteins (Boye & Alli, 2000).

Fig. 3 shows the DSC thermograms of native and PL treated WPI solutions heated from 40 to 100 °C. Untreated WPI exhibited an endothermic peak at about 77 °C. In agreement with the findings of Paulsson and Dejmek (1990), this peak represents the denaturation temperature of β-lactoglobulin, the major fraction of whey protein, and is associated to an endothermic process since denaturation involves the dissociation of intramolecular bonds (non-covalent and in some cases, covalent (disulphide) bonds).

The differences detected between the DSC profiles of untreated and PL treated samples suggest that PL treatment altered the thermal behaviour of WPI. Table 1 and Fig. 4 present DSC characteristic temperatures and denaturation enthalpy ratio of untreated and PL-treated WPI samples, as a function of the energy dose. Results show that no significant ($p>0.05$) changes in the onset temperature (58.05-56.49 °C), the peak temperature (77.55-76.64 °C), and offset temperature (91.30-92.24 °C) values and denaturation enthalpy ratio of the WPI samples were observed after PL treatment at 4 J/cm^2, as compared with the untreated samples. Instead, a significant ($p\leq0.05$) decrease was observed after PL treatment at 12 J/cm^2 for T_{offset} and T_d, and at 8 J/cm^2 for T_{onset} and ΔH_{ratio}. Further increase of the energy dose up to 16 J/cm^2 led to not significant ($p>0.05$) changes of the characteristics temperatures values (T_{offset}, T_d, T_{onset}), while a

significant *($p \leq 0.05$)* decrease of ΔH_{ratio} was detected when the energy dose was increased from 8 to 12 J/cm^2. This is likely to be attributed to the destruction of the hydrophobic bonds resulting in protein denaturation. Changes in denaturation enthalpies could be associated with changes in the bonding pattern, where a protein conformational state with fewer or weaker bonds would require less energy to unfold the molecule, which is consistent with the observed reduction of enthalpy. Decreasing of the area under the curve due UV irradiation suggested that UV irradiation decreased the portion of native protein (Muranov et al., 2011). The slight but not significant *($p > 0.05$)* increase of denaturation enthalpy ratio of WPI samples processed with PL treatments at 16 J/cm^2 could be correlated to a possible reformation of intra and intermolecular hydrophobic bonds in the resulting disordered protein molecules (Chandrapala et al., 2011).

In conclusion, the DSC graphs show combination of endothermic reactions, such as the disruption of hydrogen bonds, and exothermic processes, such as the disruption of hydrophobic interactions (Van der Plancken, Van Loey, Hendrickx, 2006). According to these observations, the DSC analyses of the WPI samples clearly demonstrate that the modification of protein structure and the structure loss are induced by PL treatments.

3.3 Changes in the primary structure: determination of carbonyl groups

Carbonyls could result from oxidative scission of peptides, which are transformed into carbonyl groups during protein oxidation (Stadtman, 1993). Therefore, the detection of carbonyl concentration may be used as an indirect measurement of irreversible modification of protein primary structure (Fenaille et al., 2006).

Fig. 5 shows the carbonyls concentrations of untreated and PL treated WPI samples as a function of the energy dose. The untreated WPI samples contained 3.94 nmol carbonyls/ mg proteins. The exposure to PL treatments at 4, 8, 12, and 16 J/cm^2 significantly *($p \leq 0.05$)* increased the protein carbonyl content compared with the untreated sample, which rose up to 4.39, 4.99, 5.13 and 5.39 nmol/mg, respectively. It is known that amino acids with NH or NH_2 groups on their side chains are very sensitive to oxidation, and these groups are transformed in carbonyl groups during protein oxidation (Sante-Lhoutellier, Aubry, Gatellier, 2007). Therefore, when WPI samples were irradiated by PL, carbonyls were formed due to the modification of covalent bonds. These findings are consistent with those previously observed on the formation of protein carbonyls by photo-oxidation or thermal treatments in dairy product processing. Scheidegger et al. (2010) found that protein carbonyl content increased as a function of UV or Fluorescent irradiation time

for both whole and skim milk due to photo-oxidation. In addition, Viljanen, Kylli, Hubbermann, Schwarz and Heinonen (2005) also reported that photo-oxidation affects the primary structure of proteins producing changes in individual amino acids as carbonyl formation and loss of aromatic amino acids for several complex food matrices.

3.4 Changes in protein functionality

3.4.1 Protein Solubility

Solubility is an important characteristic for the utilization of proteins in food systems and a prerequisite for derived functional properties like emulsification, foaming and gelation (Pearce & Kinsella, 1978).

The solubility of untreated and PL irradiated WPI samples as a function of the energy doses is shown in Fig. 6. While the solubility of samples processed at 4 J/cm^2 was not significantly ($p>0.05$) different from that of the untreated sample, a significant ($p\leq0.05$) increase of protein solubility was observed instead for the samples treated at energy doses from 8 to 16 J/cm^2. The solubility reached its maximum value for samples treated at 16 J/cm^2 (92%). These results are consistent with the previous observations of the effects of PL treatments on proteins structures.

In fact, due to the exposure to PL treatments, WPI samples were partially unfolded and the unfolding enhanced the release of hydrophilic amino acid residues to the protein surface and promoted the interactions between protein molecules and solute and, consequently, increased the solubility. Similarly, increasing solubility of soybean protein isolates with increasing pulsed electric field strength due to partial unfolding has been reported by Li, Chen, and Mo (2007). Jambrak, Mason, Lelas, Herceg, and Herceg (2008) stated that an increased solubility of whey protein detected after exposure to pulsed electric fields could be attributed to the changes in three-dimensional structures of globular protein, which resulted in an increase of the number of charged groups (NH_4^+,COO^-). As a result, the electrical conductivity of the treated sample was higher compared to that of the control, which would increase protein-water interactions due to the higher electrostatic forces. Elmnasser et al. (2008) also observed an increase of the polarity of β-lactoglobulin protein as well as of tryptophanyl residue microenvironment exposed to UV light, which was attributed to light-induced folding changes or to the occurrence of protein aggregation by disulphide bonds.

3.4.2 Foaming properties

Foaming properties of native and PL treated WPI samples at different energy doses, were studied by evaluating the foaming capacity and foaming stability and the results are reported in Fig. 7. Untreated samples showed a foaming capacity and foaming stability of 51 % and 153 %, respectively.

The exposure to PL with energy doses higher than 4 J/cm^2 was enhancing significantly *($p \leq 0.05$)* the foaming properties of WPI solutions, in comparison to the untreated samples. The maximum values (235 % for foaming capacity, and 81 % for foaming stability) were detected after PL treatments at 16 J/cm^2.

Fernández et al. (2012) also reported an improvement of foaming properties upon PL irradiation of β-lactoglobulin. The authors observed an increment of about 12% of the foaming capacity of 4 J/cm^2 treated β-lactoglobulin solution (10 g/L) compared to the native sample, which is consistent with results of this work in which we observed an increment of about 9% after applying a PL treatment at the same conditions.

Generally, the formation of foam is thermodynamically favourable due to the simultaneous dehydration of the hydrophobic interface and the exposition of hydrophobic portions of the protein (Dickinson, 1989). The foaming properties, in fact, mainly depend on the protein capability to form film at the air-water interface. The foam formation involves a large number of factors, including physical and chemical properties of the proteins, as well as environmental factors such as ionic strength or pH. In addition, steric and electrostatic repulsions during the adsorption processes as well as by the stability of the disulphide bonds, which stabilize the secondary and tertiary structures of the protein, are playing an important role in foam formation.

Thus, the higher values of foam capacity and stability exhibited by PL treated WPI found in this work, could be attributed to the partial denaturation induced by PL irradiation, which could increase the hydrophobicity of the treated proteins.

PL induced partial denaturation and formation of free SH-groups could increase the ability of treated sample to adsorb air bubbles more rapidly at the air/water interface. It is worth noting that foam stability is dictated by the number of exposed hydrophobic groups of the molecules at interface between foam (bubble) and air, the number of hydrophilic groups in contact with the aqueous solution and their relative proportion. Additionally, the net proton charge could influence the adsorption of proteins at the air-water interface (Cherry & McWatters, 1981). Sato and Nakamura (1977) observed that, when the net charge on egg white proteins was increased by succinylation, their foaming capacity was enhanced.

3.5 Correlation analysis

The correlation between the functional properties of untreated and PL treated WPI samples examined in this work are reported in Table 2. Data confirmed the observation reported in the previous paragraphs. Strong positive correlations were observed among free SH-groups content, carbonyl groups concentration and protein functional properties (solubility and foaming). These results clearly indicated that there is a strong relationship between the changes detected in the native proteins, namely of their primary, secondary and tertiary structure, and the improvement of their functional properties detected. A strong negative correlation between the number of free SH-groups and denaturation enthalpy was instead observed. It can be hypothesized that the formation of free SH-groups and the consequent destabilization of the conformational structure due to the breaking of disulphide bridges were causing the decrease of the denaturation enthalpy, if any. Therefore, these results indicated that the increase of SH groups concentrations consequent to PL irradiation has a strong correlation with the physicochemical properties governing the overall functionality of WPI samples.

4. Conclusions

The results of this study highlighted the potentiality of PL technology to induce changes of WPI conformational and functional properties in a dose depending manner. The exposure to light treatments led to the increase of the concentration of SH-groups and the formation of a carbonyl group, which suggested the modification of the protein tertiary and primary structure. Small changes of whey proteins secondary structure were also confirmed by FT-IR tests. Solubility and functional properties were significantly *($p \leq 0.05$)* improved by applying PL treatments at energy doses in the range from 8 to 12 J/cm^2. The analysis of data of denaturation enthalpies revealed that, as a consequence of the exposure to PL, significant reductions of enthalpy and denaturation temperature of WPI occurred. All these results supported the hypothesis that PL treatments were able to partially unfold WPI samples. This partial unfolding of whey proteins exposed the hydrophobic groups, buried inside the structure in the native proteins, to the surface and allowed to increase their functional properties such as solubility and foaming capacity.

References

1. Barth, A. (2007). Infrared spectroscopy of proteins. *Biochimica et Biophysica Acta, 1767,* 1073–1101.
2. Beveridge, T., Toma, S.J., & Nakai, S. (1974). Determination of SH- and SS-groups in some food proteins using Ellman's reagent. *Journal of Food Science, 39,* 49-53.
3. Bouaouina, H., Desrumaux, A., Loisel, C., & Legrand, J. (2006). Functional properties of whey proteins as affected by dynamic high-pressure treatment. *International Dairy Journal, 16,* 275–284.
4. Boye, J. I., & Alli, I. (2000). Thermal denaturation of mixtures of α-lactalbumin and β-lactoglobulin: a differential scanning calorimetric study. *Food Research International, 33,* 673-682.
5. Brew, K. (2003). α-Lactalbumin. In P. F. Fox, & P. L. H. McSweeney (Eds.), Advanced dairy chemistry 1. Proteins (Part A) (pp. 387-412). New York: Kluwer Academic/Plenum Publishers.
6. Byler, D. M., & Purcell, J. M. (1989). FTIR examination of thermal denaturation and gel-formation in whey proteins. Conference- Proceedings. 7th International Conference on Fourier Transform Spectroscopy (December 1, 1989).
7. Byler, D.M., & Susi, H. (1986). Examination of the secondary structure of proteins by deconvoluted FTIR spectra. *Biopolymers, 25,* 469-487.
8. Chandrapala, J., Zisu, B., Palmer, M., Kentish, S., & Ashokkumar, M. (2011). Ultrasonicssono chemistry effects of ultrasound on the thermal and structural characteristics of proteins in reconstituted whey protein concentrate. *Ultrasonics Sonochemistry, 18,* 951–957.
9. Cherry, J., & McWatters, K. (1981). Whippability of foods. In J. P. Cherry (Ed.), Protein functionality in foods (pp. 149-176). Washington D.C.: American Chemical Society.
10. Chobert, J M. (2012). Milk protein tailoring to improve functional and biological properties. *Jornal of BioScience and Biotechnology, 1,* 171-197.
11. Davis, J.P., Doucet, D., & Foegeding, E. A. (2005). Foaming and interfacial properties of hydrolyzed β-lactoglobulin. *Journal of Colloid and Interface Science, 288,* 412-422.
12. Dickinson, E. (1989). Food colloids — an overview. *Colloids and Surfaces, 42,* 191–204.
13. Dissanayake, M., & Vasiljevic, T. (2009). Functional properties of whey proteins affected by heat treatment and hydrodynamic high-pressure shearing. *Journal of Dairy Science, 92,* 1387–1397.

525 14. Dissanayake, M., Ramchandran, L., Piyadasa, C., & Vasiljevic, T. (2013). Influence of heat
526 and pH on structure and conformation of whey proteins. *International Dairy Journal 28,* 56-
527 61.
528 15. Ellman, G.L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, *82,*
529 70-77.
530 16. Elmnasser, N., Dalgalarrondo, M., Orange, N., Bakhrouf, A., Haertle, T., Federighi, M., &
531 Chobert, J. (2008). Effect of pulsed-light treatment on milk proteins and lipids. *Journal of*
532 *Agricultural and Food Chemistry, 56,* 1984-1991.
533 17. Enomoto, H., Li, C. P., Morizane, K., Ibrahim, H.R., Sugimoto, Y., Ohki, S., Ohtomo, H., &
534 Aoki, T. (2007). Glycation and phosphorylation of β-lactoglobulin by fry-heating: effect on
535 protein structure and some properties. *Journal of Agricultural and Food Chemistry, 55*,
536 2392-2398.
537 18. Fabian, H., & Mäntele, W. (2002). Infrared Spectroscopy of Proteins. In J. M. Chalmers & P.
538 R. Griffiths (Eds.), Handbook of Vibrational Spectroscopy (pp 3999-3424). Chichester: John
539 Wiley and Sons.
540 19. Fenaille, F., Parisod, V., Visani, P., Populaire, S., Tabet, J., & Guy. P. A. (2006).
541 Modifications of milk constituents during processing: A preliminary benchmarking study.
542 *International Dairy Journal, 16,* 728–739.
543 20. Fernández, E., Artiguez M.L., de Marañón I. M., Villate, M., Blanco, F.J., Arboleya, J.C.
544 (2012). Effect of pulsed-light processing on the surface and foaming properties of β-
545 lactoglobulin. *Food Hydrocolloids, 27,* 154-160.
546 21. Hambling, S. G., McAlpine, A. L, Sawyer, L. (1992). β-Lactoglobulin. In P. F. Fox (Ed.),
547 Advanced dairy chemistry 1. Proteins (pp 141-190). London: Elsevier Applied Science.
548 22. Hardham, H. F. (1981). The determination of total and reactive sulfhydryl of whey protein
549 concentrates. *Australian Journal of Dairy Technolgy, 36,* 153-155.
550 23. Hoffmann, M. A. M & van Mil, P. J. J. M. (1999). Heat-Induced Aggregation of β-
551 Lactoglobulin as a Function of pH. *Journal of Agricultural and Food Chemistry, 47,* 1898-
552 1905.
553 24. Jambrak, A. R., Mason, T. J., Lelas, V., Herceg, Z., & Herceg, I. L. (2008). Effect of
554 ultrasound treatment on solubility and foaming properties of whey protein suspensions.
555 *Journal of Food Engineering, 86,* 281–287.
556 25. Kehoe, J. J., Remondetto, G.E., Subirade, M., Morris, E.R., & Brodkorb, A. 2008.
557 Tryptophan-mediated denaturation of beta-lactoglobulin A by UV irradiation. *Journal of*
558 *Agricultural and Food Chemistry, 56,* 4720–4725.

559 26. Kim, J., Saltmarch,M., & Labuzu, T. P. (1981). Non-enzymatic browning of hygroscopic
560 whey powders in open versus sealed pouches. *Journal of Food Processing and Preservation,*
561 *5,* 49–57.
562 27. Kinsella, J. E. (1982). Protein structure and functional properties: emulsification and flavor
563 binding effects. In J. P. Cherry (Ed.), Food Protein Deterioration, Mechanisms and
564 Functionality (p 301). Washington, D.C.: American Chemical Society.
565 28. Krimm, S., & Bandekar, J. (1986). Vibrational spectroscopy and conformation of peptides,
566 polypeptides, and proteins. *Advances in Protein Chemistry, 38,* 181−364.
567 29. Kristo, E., Hazizaj, A., & Corredig,M. (2012). Structural changes imposed on whey proteins
568 by UV irradiation in a continuous UV light reactor. *Journal of Agricultural and Food*
569 *Chemistry, 60,* 6204–6209.
570 30. Lasagabaster, A., Arboleya, J. C., & de Marañón, I. M. (2011). Pulsed light technology for
571 surface decontamination of eggs: impact on Salmonella inactivation and egg quality.
572 *Innovative Food Science & Emerging Technologies, 12,* 124-128.
573 31. Lee, S., Morr, C.V. & Ha, E. Y. W. (1992). Structural and functional properties of caseinate
574 and whey protein isolate as affected by temperature and pH. *Journal of Food Science, 57,*
575 1210-1214.
576 32. Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A.G., Ahn, B. W.,
577 Shaltiel, S., & Stadtman, E. R. (1990). Determination of carbonyl content in oxidatively
578 modified proteins. *Methods in Enzymology, 186,* 464–478.
579 33. Li, Y., Chen, Z., & Mo, H. (2007). Effects of pulsed electric fields on physicochemical
580 properties of soybean protein isolates. *Lebensmittel-Wissenschaft & Technologie, 40,* 1167–
581 1175.
582 34. Lowry, O. H., Rosebrough, N. J., Lewis, A. L., & Randall, R. J. (1951). Protein measurement
583 with the Folin phenol reagent. *Journal of Biological Chemistry, 193,* 265–275.
584 35. McClements, D. J. (1999). Food Emulsions: Principles, Practice and Techniques. Boca
585 Raton, Florida, CRC press.
586 36. Muranov, K. O., Maloletkina, O. I., Poliansky, N. B., Markossian, K. A., Kleymenov, S. Y.,
587 Rozhkov, S. P., Goryunov, A. S., Ostrovsky, M. A., & Kurganov, B. I. (2011). Mechanism of
588 aggregation of UV-irradiated β_L-crystallin. *Experimental Eye Research, 92,* 76-86.
589 37. Neves-Petersen, M. T., Gryczynski, Z., Lakowicz, J., Fojan, P., Pedersen, S., Petersen, E. &
590 Petersen, S. B. (2002). High probability of disrupting a disulphide bridge mediated by an
591 endogenous excited tryptophan residue. *Protein Science*, *11,* 588–600.

592 38. Nicorescu, I., Loisel, C., Riaublanc, A., Vial, C., Djelveh, G., Cuvelier, G., & Legrand, J.
593 (2009). Effect of dynamic heat treatment on the physical properties of whey protein foams.
594 *Food Hydrocolloids, 23,* 1209–1219.
595 39. Oms-Oliu, G., Martin-Belloso, O., & Soliva-Fortuny, R. (2010).Pulsed Light Treatments for
596 Food Preservation. A Review. *Food and Bioprocess Technology, 3,* 13-23.
597 40. Pataro, G., Sinike, M., Capitoli, M. M., Donsì, G., Ferrari, G. (2015). The influence of post-
598 harvest UV-C and pulsed light treatments on quality and antioxidant properties of tomato
599 fruits during storage. *Innovative Food Science and Emerging Technologies, 30,* 103–111.
600 41. Parris, N., Purcell, J.M., & Ptashkin, S.M. (1991). Thermal denaturation of whey proteins in
601 skim milk. *Journal of Agricultural and Food Chemistry, 39,* 2167–2170.
602 42. Pearce, K.N. & Kinsella, J. E. (1978). Emulsifying properties of proteins: evaluation of a
603 turbidimetric technique. *Journal of Agricultural and Food Chemistry, 26,* 716-723.
604 43. Permyakov, E. A., Permyakov, S. E., Deikus, G. Y., Morozova- Roche, L. A., Grishchenko,
605 V. M., Kalinichenko, L. P., & Uversky, V. N. (2003). Ultraviolet illumination-induced
606 reduction of α-lactalbumin disulfide bridges. *Proteins: Structure, Function, Genetics, 51,*
607 498−503.
608 44. Paulsson, M., & Dejmek. P. (1990). Thermal denaturation of whey proteins in mixtures with
609 caseins studied by differential scanning calorimetry. *Journal of Dairy Science, 73,* 590–600.
610 45. Qin, Z., Guo, X., Lin, Y., Chen, J., Liao, X., Hu, X., & Wu, J. (2013). Effects of high
611 hydrostatic pressure on physicochemical and functional properties of walnut (*Juglans regia*
612 L.) protein isolate. *Journal of the Science of Food and Agriculture*; *93,* 1105–1111.
613 46. Sante-Lhoutellier, V., Aubry, L., & Gatellier, P. (2007). Effect of oxidation on in vitro
614 digestibility of skeletal muscle myofibrillar proteins. *Journal of Agricultural and Food*
615 *Chemistry. 55,* 5343–5348.
616 47. Sato, Y., & Nakamura, R. (1977). Functional properties of acetylated and succinylated egg
617 white. *Agricultural and Biological Chemistry, 41,* 2163-2168.
618 48. Scheidegger, D., Pecora, R.P., Radici, P.M., and Kivatinitz, S.C. (2010). Protein oxidative
619 changes in whole and skim milk after ultraviolet or fluorescent light exposure. *Journal of*
620 *Dairy Science, 93,* 5101–5109.
621 49. Segat, A., Misra, N. N., Fabbro, A., Buchini, F., Lippe, G., Cullen, P. J., & Innocente, N.
622 (2014). Effects of ozone processing on chemical, structural and functional properties of whey
623 protein isolate. *Food Research International, 66*, 365–372.
624 50. Stadtman E. R. (1993). Oxidation of free amino acids and amino acid residues in proteins by
625 radiolysis and by metal-catalyzed reactions. *Annual Reviews of Biochemistry, 62,* 797-821.

626 51. Sun, W. (2010). Effects of composition and oxidation of proteins on their solubility
627 aggregation and proteolytic susceptibility during processing of cantonese sausage. *Food*
628 *Chemistry, 124,* 336-341.
629 52. Surewicz, W. K., Szabo, A. G., Mantsch. H. H. (1987). Conformational properties of azurin
630 in solution as determined from resolution-enhanced Fourier transform infrared spectra.
631 *European Journal of Biochemistry, 167,* 519–523.
632 53. Ustunol, Z. (2015). Physical, chemical and Processing-induced changes in proteins. In Z.
633 Ustunol (Ed.), Applied Food Protein Chemistry (pp 23-46). Chichester: John Wiley and
634 Sons.
635 54. Van der Plancken, I., Van Loey, A., & Hendrickx, M. E. G. (2006). Effect of heat treatment
636 on the physico-chemical properties of egg white proteins: a kinetic study. *Journal of Food*
637 *Engineering, 75,* 316-326.
638 55. Verheul, M., Roefs, S. P. F. M., Mellema, J., & de Kruif, K. G. (1998). Power law behavior
639 of structural properties of protein gels. *Langmuir, 14,* 2263–2268.
640 56. Viljanen, K., Kylli, P., Hubbermann, E., Schwarz, K., & Heinonen, M. (2005). Anthocyanin
641 antioxidant activity and partition behaviour in whey protein emulsion. *Journal of*
642 *Agricultural and Food Chemistry, 53,* 2022–2027.
643 57. Wekhof, A. (2000). Disinfection with flash lamps. *Journal of Pharmaceutical Science and*
644 *Technology, 54,* 264-276.
645 58. Xiang, B.Y., Ngadi, M.O., Ochoa-Martinez, L., & Simpson, M.V. (2009). Pulsed electric
646 field induced structural modification of whey protein isolate. *Food and Bioprocess*
647 *Technology, 4,* 1341–1348.
648 59. Yang, S., Kong, J., Dong, A., & Yu, S. (2015). Obtaining information about protein
649 secondary structures in aqueous solution using Fourier transform IR spectroscopy. *Nature*
650 *Protocols, 10,* 382–396.
651 60. Zhu, H., & Damodaran, S. (1994). Heat-Induced Conformational Changes in Whey Protein
652 Isolate and Its Relation to Foaming Properties. *Journal of Agricultural and Food Chemistry,*
653 *42,* 846-855.
654

655 **Fig. captions**

656 **Fig. 1** Total and free SH group content (μmolSH/g) of untreated (0 J/cm^2) and PL treated WPI
657 solutions at different energy doses. Means with different lowercase letters within the same
658 sample are significantly different ($p \leq 0.05$).

659 **Fig. 2** Fourier transform infrared spectra of untreated and PL treated WPI samples (10 mg/mL).
660 Each spectrum is represented as second derivative of the original spectra. (a) Untreated (0
661 J/cm^2), (b), (c), (d) and (e) PL treated samples at energy doses of 4, 8, 12 and 16 J/cm^2,
662 respectively.

663 **Fig. 3** DSC thermograms of untreated and PL treated WPI solutions. (a) Untreated (0 J/cm^2),
664 (b), (c), (d) and (e) PL treated samples at energy doses of 4, 8, 12 and 16 J/cm^2, respectively.

665 **Fig. 4** Denaturation enthalpy ratio (ΔH_{ratio}) of untreated (0 J/cm^2) and PL treated WPI samples at
666 different energy doses. Means with different lowercase letters are significantly different ($p \leq$
667 0.05).

668 **Fig. 5** Carbonyl groups concentration (nmol/mg protein) of untreated (0 J/cm^2) and PL treated
669 WPI samples at different energy doses. Means with different lowercase letters are significantly
670 different ($p \leq 0.05$).

671 **Fig. 6** Protein solubility of untreated (0 J/cm^2) and PL treated WPI samples at different energy
672 doses. Means with a different lowercase letters are significantly different ($p \leq 0.05$).

673 **Fig. 7** Comparison of foaming properties of untreated (0 J/cm^2) and PL treated WPI samples at
674 different energy doses. Means with different lowercase letters within the same sample are
675 significantly different ($p \leq 0.05$).

Figure

Fig. 1

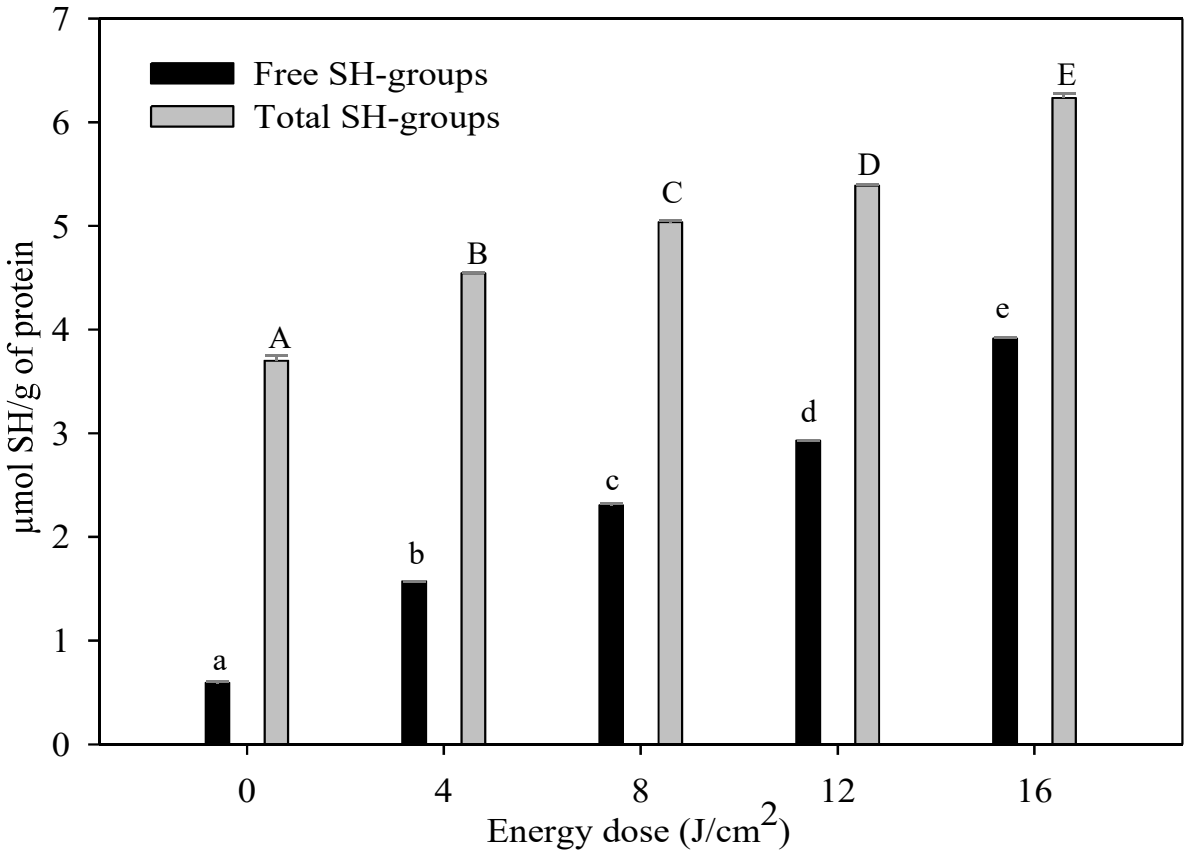
Free SH-groups
Total SH-groups
µmol SH/g of protein
Energy dose (J/cm2)
0
1
2
3
4
5
6
7
0
4
8
12
16
a
A
b
B
c
C
d
D
e
E

Fig. 2

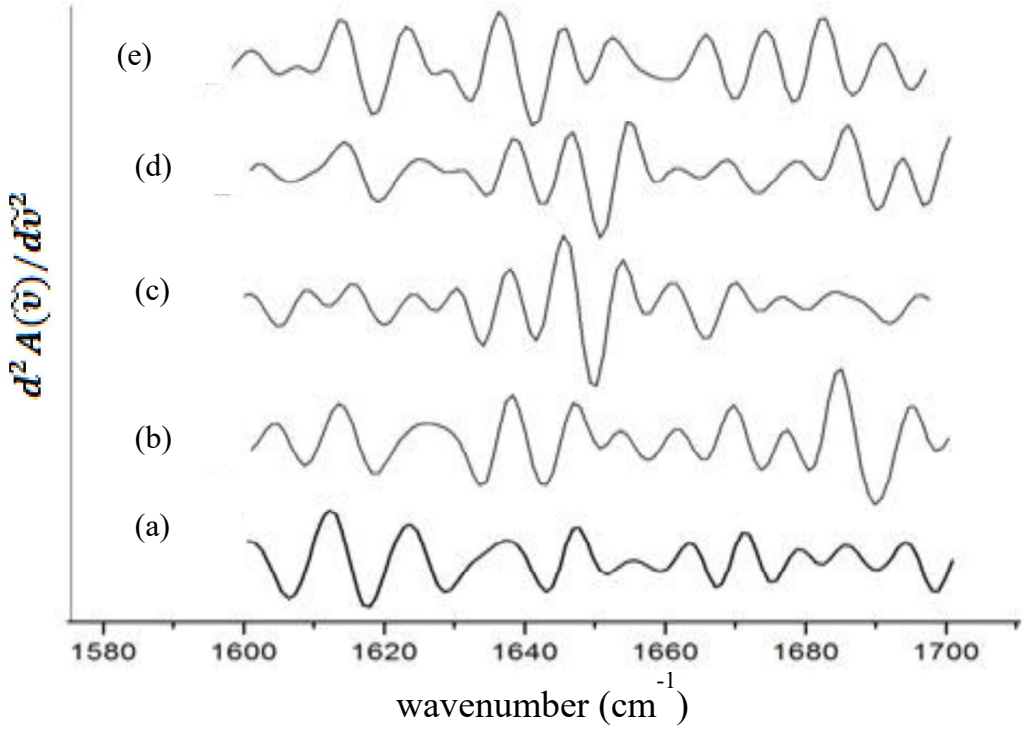
(e)
(d)
(c)
(b)
(a)
$d^2A(\tilde{\nu})/d\tilde{\nu}^2$
1580
1600
1620
1640
1660
1680
1700
wavenumber (cm^{-1})

Fig. 3

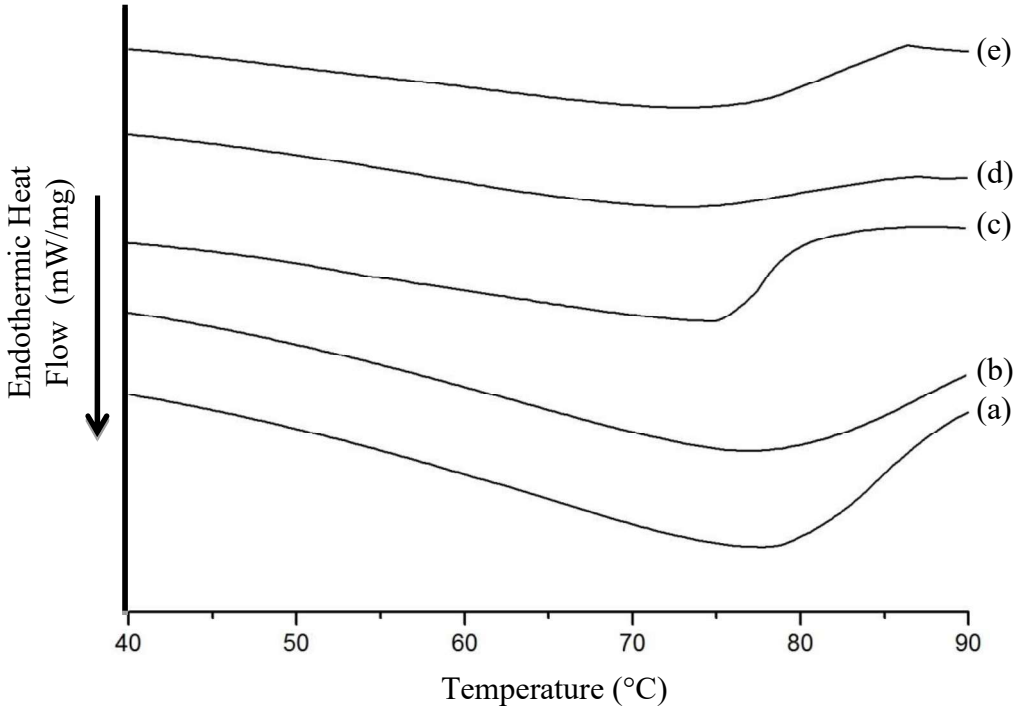
Endothermic Heat
Flow (mW/mg)
(e)
(d)
(c)
(b)
(a)
40
50
60
70
80
90
Temperature (°C)

Fig. 4

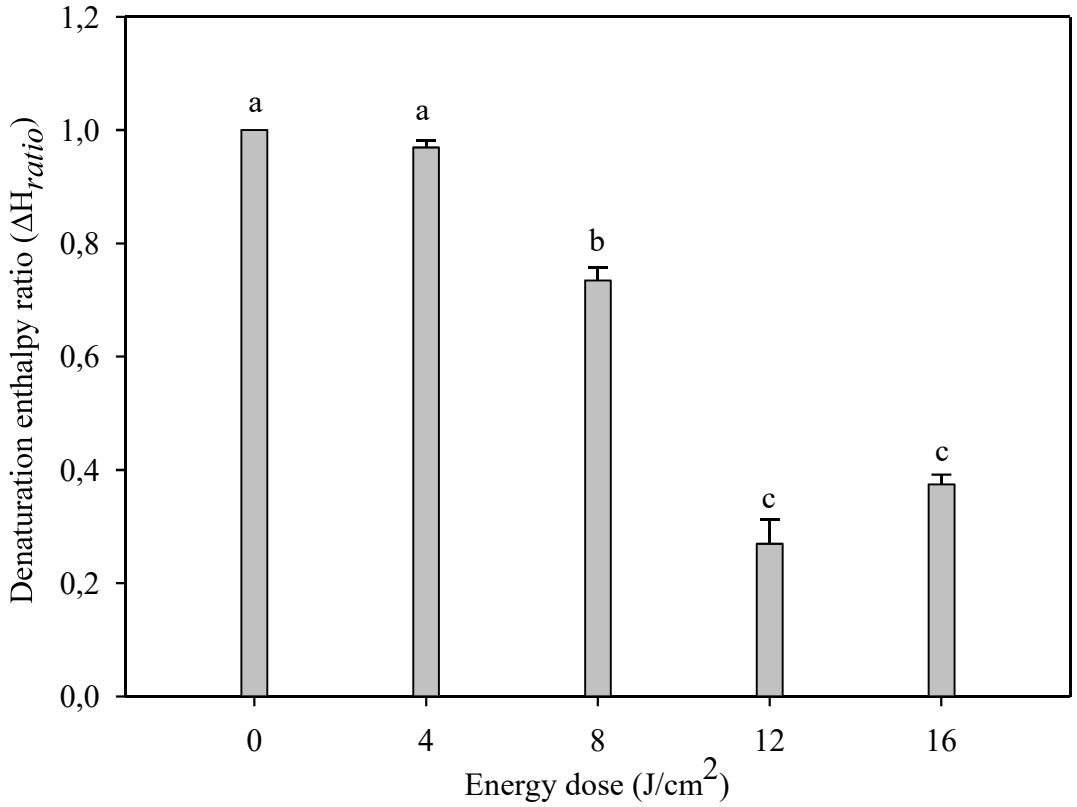
1,2
1,0
0,8
0,6
0,4
0,2
0,0
Denaturation enthalpy ratio (ΔH_{ratio})
a
a
b
c
c
0
4
8
12
16
Energy dose (J/cm^2)

Fig. 5

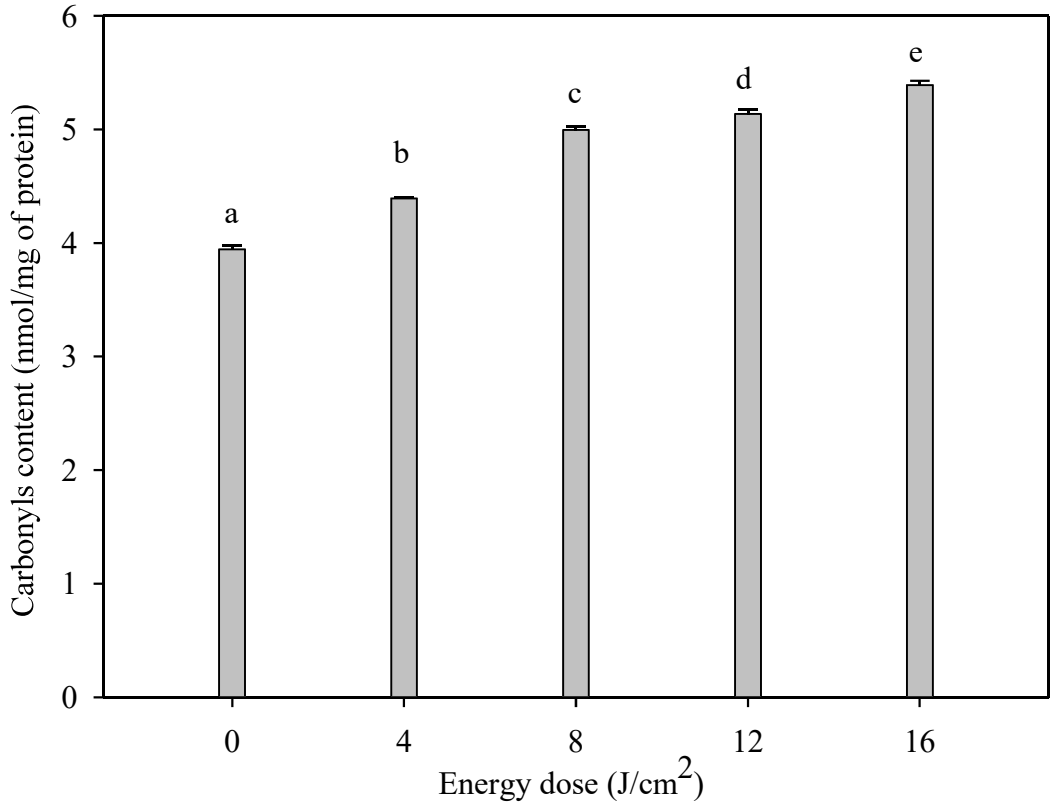
Carbonyls content (nmol/mg of protein)
0
1
2
3
4
5
6
a
b
c
d
e
0
4
8
12
16
Energy dose (J/cm2)

Fig. 6

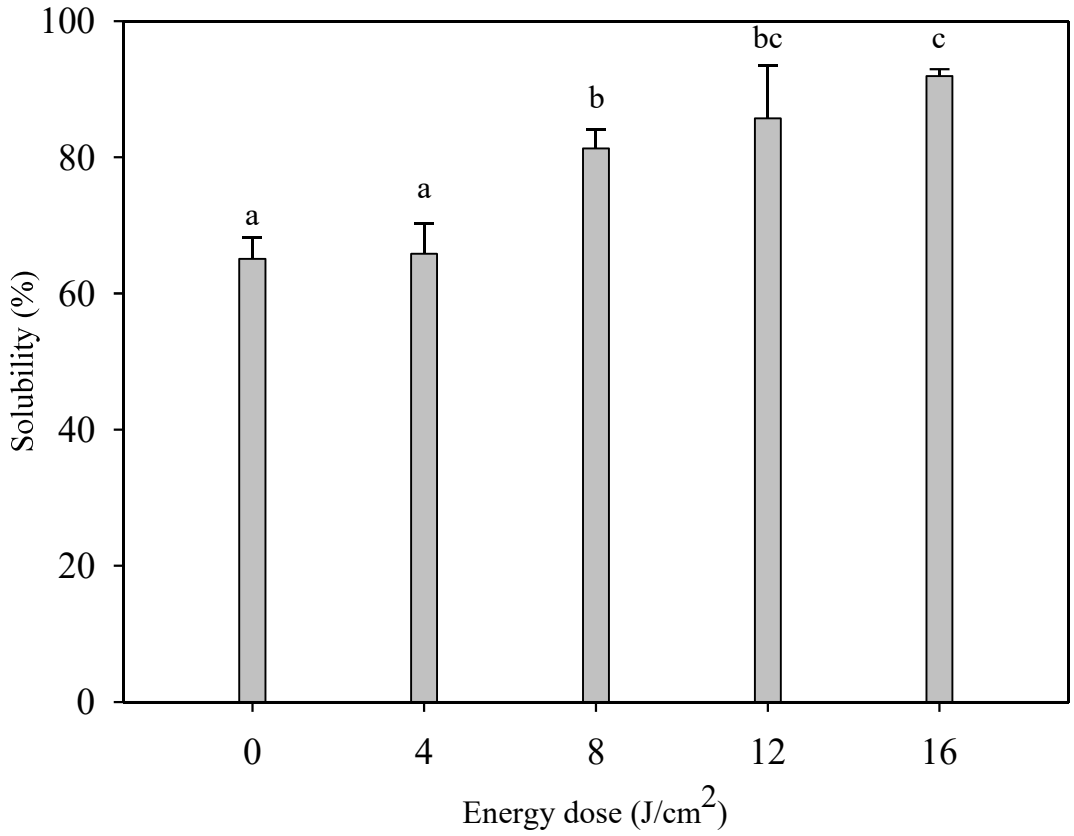

Fig. 7

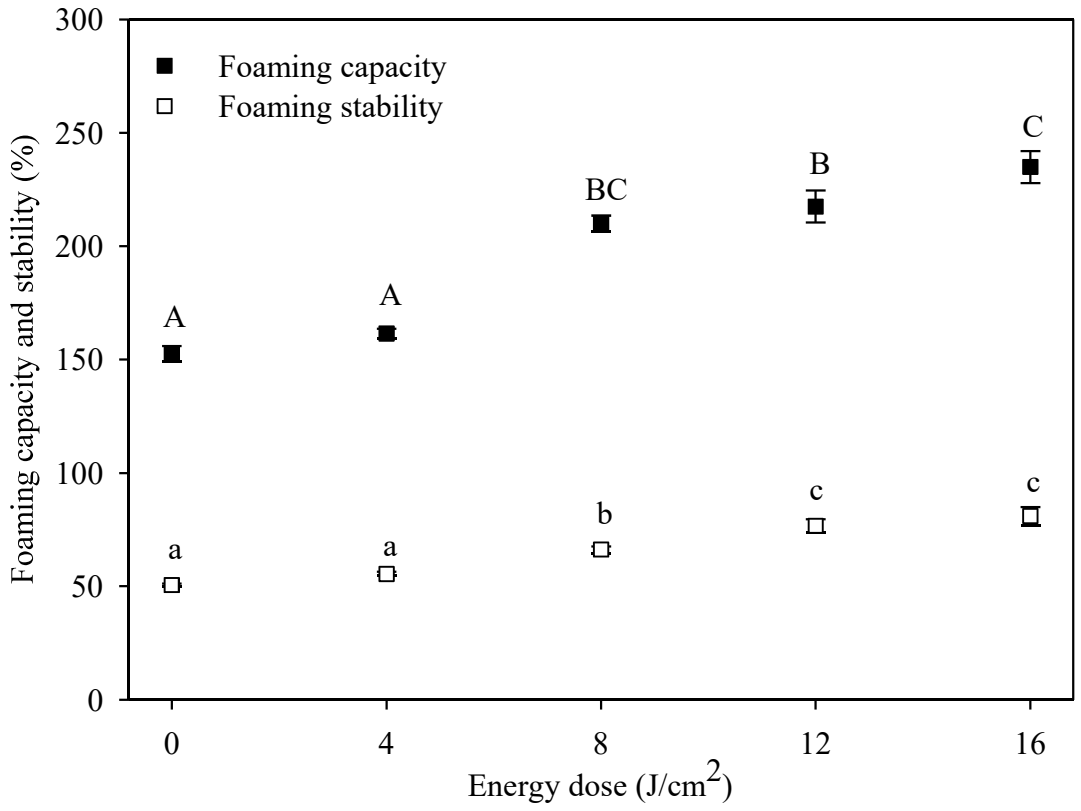
Foaming capacity
Foaming stability
Foaming capacity and stability (%)
Energy dose (J/cm2)
300
250
200
150
100
50
0
0
4
8
12
16
A
A
BC
B
C
a
a
b
c
c

Table 1. DSC characteristic temperatures of untreated (0 J/cm^2) and PL treated at different energy doses (4–16 J/cm^2) WPI samples. Means with different superscript letters in each column are significantly different ($p \leq 0.05$).

Energy Dose (J/cm^2)	**T_{onset} (°C)**	**T_d (°C)**	**T_{offset} (°C)**
0	57.0^{a}	76.9^{a}	92.3^{a}
4	56.3^{ab}	76.7^{a}	91.9^{ab}
8	54.6^{ab}	74.0^{ab}	88.9^{bc}
12	53.7^{b}	73.0^{b}	86.4^{c}
16	54.0^{b}	71.3^{b}	86.4^{c}

Table 2. Correlation coefficient among the functional properties of untreated and after PL treated (4-16 J/cm^2) WPI samples.

WPI properties	**Free SH-groups**	**Carbonyls content**	**Denaturation enthalpy**	**Solubility**	**Foaming capacity**	**Foaming stability**
Free SH-groups	1.000	0.945	-0.823	0.946	0.923	0.964
Carbonyls content	0.945	1.000	-0.856	1.000	0.998	0.967
Denaturation enthalpy	-0.823	-0.856	1.000	-0.870	-0.844	-0.939
Solubility	0.946	1.000	-0.870	1.000	0.997	0.973
Foaming capacity	0.923	0.998	-0.844	0.997	1.000	0.953
Foaming stability	0.964	0.967	-0.939	0.973	0.953	1.000

*Graphical Abstract
Click here to download high resolution image