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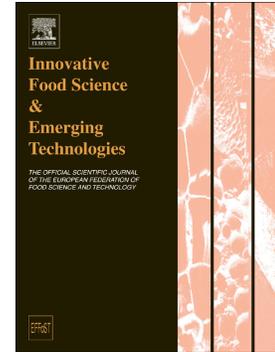
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## Accepted Manuscript

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## Electric field effects on $\beta$ -lactoglobulin thermal unfolding as a function of pH – impact on protein functionality

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**Abstract**

The presence of moderate electric fields (MEF) during ohmic heating (OH) treatment of whey protein systems have demonstrated potential to change physicochemical and functional properties, like aggregation rate and extension or viscoelastic behaviour. However, the specific action of MEF upon the molecular structure of proteins, particularly during thermal processing has yet to be clarified. The effects of MEF in pure fractions of  $\beta$ -lactoglobulin ( $\beta$ -lg) under non-aggregating conditions (low concentration and ionic strength), were investigated in this work. Protein samples were identically heat-treated through conventional and OH methods and at different pH values.  $\beta$ -lg's structural features were characterized by evaluation of secondary structure distribution and local conformational changes using techniques such as circular dichroism, intrinsic and extrinsic fluorescence and free thiol groups reactivity. It was confirmed that MEF affects  $\beta$ -lg upon thermal unfolding, resulting in distinctive structural features, surface hydrophobicity and SH reactivity. The mechanism of action is probably related with the molecular motion induced by the oscillating electric field and is more pronounced at neutral pH, where  $\beta$ -lg is more susceptible to thermal structural changes. These results contribute to a better understanding of OH processing and its effects in food matrices reinforce the possibility of using MEF as a tool to change protein functionality.

**Keywords**

$\beta$ -lactoglobulin, Moderate Electric Fields, Ohmic Heating, Protein Denaturation, Protein Functionalization

## Introduction

Whey proteins have many applications in food industry and technology, either as ingredients or as functional agents.  $\beta$ -lactoglobulin ( $\beta$ -lg) is the major fraction in whey protein ingredients and dominates their functional properties, however other constituents, especially other proteins, are also playing an important role in the complex interactions that can be formed (Petit et al., 2016). This small globular protein with 162 amino acids contains a central barrel, formed by 8  $\beta$ -strands, with a ninth  $\beta$ -strand and a 3-turn  $\alpha$ -helix on the exterior (Kontopidis, Holt, & Sawyer, 2004).  $\beta$ -lg presents a dynamic structural behaviour strongly governed by the environmental pH, which influences the monomer-dimer equilibrium, access to the central hydrophobic barrel or exposure of surface hydrophobic sites (Collini et al., 2003; Taulier & Chalikian, 2001). Furthermore, by controlling other physicochemical factors, such as protein concentration, ionic strength, temperature or pressure, it is possible to tailor the properties and functionality of whey protein systems (Bryant & McClements, 1998). In reason of these properties, whey proteins can be used e.g. as emulsifiers, stabilizers, gelation agents, building blocks for micro/nano-particles and carriers, edible films and coatings.

Often the functionalization of proteins is achieved through denaturation, as the unfolding of the proteins results on exposure of its hydrophobic groups and free thiols (de Wit, 2009). The exposure of these groups increases protein reactivity towards the formation of new intra- and inter-molecular interactions, establishing the basis for aggregation and network formation (Nicolai, Britten, & Schmitt, 2011). Thermal denaturation is by far the predominant method to achieve protein functionalization; this strategy has been extensively reviewed and commonly used in the food industry. Ohmic heating (OH) has been established as an emerging technology, capable of providing fast, homogeneous and a highly energy efficient (> 95 %) heating. The OH process consists on the passage of electric current through a semi-conductive material (e.g. food or other biological matrix); once the material offers resistance, heat is generated directly on it (Knirsch, Alves dos Santos, Martins de Oliveira Soares Vicente, & Vessoni Penna, 2010; Sakr & Liu, 2014). OH generally operates under the specifications of moderate electric fields (MEF),

namely voltage between 1 and 1000 V, typical sinusoidal wave and broad frequency range (1 Hz to MHz range) (Sastry, 2008). Several works have been exploring MEF effects on the properties of whey protein network systems (Pereira et al., 2016; Pereira, Teixeira, & Vicente, 2011; Rodrigues et al., 2015). It was demonstrated that MEF can interfere with aggregation kinetics and gelation of whey proteins, possibly due to conformational disturbances. However, the way how the EF interacts with protein structure is far from being understood. In fact, all these works were carried in complex (i.e. WPI) and highly concentrated protein systems, which favour extensive aggregation during thermal treatment. These conditions do not allow disclosing the electrical effects involved, because complex interactions between one or several different proteins occurring during thermal aggregation can hinder structural transitions or local conformational changes. Actually it is not clear if MEF effects are the result of protein conformational changes or of impairment of the aggregation process due to electrostatic disturbances (Rodrigues et al., 2015). It is therefore necessary to study MEF effect on pure fractions of proteins, in conditions that allow confirming their influence on protein denaturation and conformation.

The objective of this work was to evaluate the effect of MEF during OH of purified fractions of  $\beta$ -lg at different physicochemical conditions of pH and temperature. It is expected that under low aggregation conditions and without the influence of other whey constituents, the impact of MEF on proteins' structural features upon thermal denaturation can be more comprehensively elucidated.

## **Materials and methods**

### **Protein solutions**

$\beta$ -lg was obtained by purifying commercial whey protein isolate (Lacprodan DI-9212, Arla Foods, Viby, Denmark) by the salting out method. The procedure of Maté and Krochta (1994) was adapted according to Konrad, Lieske and Faber (2000) (Konrad, Lieske, & Faber, 2000;

MATÉ & KROCHTA, 1994) and the product obtained was finally freeze-dried. Purity and conformation was checked against a commercial  $\beta$ -lg from Sigma-Aldrich (lyophilized powder  $\geq 90$  % PAGE) by HPLC, SDS-PAGE and circular dichroism (CD), matching it in all criteria.

$\beta$ -lg solutions at 100  $\mu$ M were prepared in 25 mM sodium phosphate buffer at pH values of 7.0, 5.7, 4.3 and 3. The solutions were stirred for 2 h to ensure full solubilisation and then the pH was checked and adjusted if needed with 1 M hydrochloric acid or sodium hydroxide. The conditions established were based on preliminary tests, aiming to minimize protein aggregation and optimize the analytic technique used.

### **Thermal treatments**

Thermal treatments were performed at target temperatures of 50, 60, 70, 80 and 90 °C for a total of 10 min. The treatment time included the come-up time (5 to 7 min according to the target temperature) and the holding time for the remaining period. Additionally fractions of  $\beta$ -lg solutions, unheated and not exposed to MEF, were used as control solutions in all determinations. The electric field applied ranged from 80 V/cm during heating to 20 V/cm during holding. The electric frequency was set at 20 kHz, once the use of frequencies on the kHz range (i.e. <17 kHz) effectively eliminates the electrochemical effects as electrolysis and electrode oxidation (Jaeger et al., 2016; Pataro et al., 2014). To evaluate the influence of MEF on protein unfolding, Cov heating treatments without presence of electric field were performed under the same heating conditions in order to evaluate non-thermal effects of OH. At the end of the treatments, samples were transferred to screw cap glass tubes and placed in a melting ice bath for 15 min. All determination were performed at room temperature and thus characterize the refolded form of the protein after being subjected to thermos-electric treatments.

### **Heating unit**

Experiments were conducted on a double-jacketed glass cylinder containing stainless steel electrodes at each edge, as described by Pereira *et al.* 2010 (Pereira, Souza, Cerqueira, Teixeira, & Vicente, 2010). For the OH treatments, the temperature was controlled by regulating the

voltage output of a function generator (Agilent 33220A, Penang, Malaysia) and then amplified on an amplifier system (Peavey CS3000, Meridian, MS, USA). For conventional treatments (Cov) the temperature was controlled by circulating water on the reactor's jacket with a circulating thermo-stabilized water bath. Temperature was measured with a type K thermocouple (Omega Engineering, Inc., Stamford, CT, USA), connected to a data logger (USB-9161, National Instruments Corporation, Austin, TX, USA). During the treatments the samples were gently stirred (with a magnetically stirrer) to ensure homogeneity.

### **Fluorescence determinations**

Fluorescence determinations were performed on the fluorescence instrument Aqualog (HOBIBA-Jobin Yvon, Inc. Japan). Intrinsic fluorescence of  $\beta$ -lg was determined by exciting tryptophan (Trp) on the samples at 295 nm and recording the emission from 300 to 450 nm. Hydrophobic sites accessibility was determined using 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescent probe. The ANS stock solution (1.5 mM) was prepared in methanol and stored at 4 °C, protected from light. For the determinations, 100  $\mu$ L of ANS stock solution were added to 3 ml of protein solution, homogenized and allowed to stabilise for 30 min at room temperature. ANS-protein complex fluorescence was determined by exciting the sample at 370 nm and recording the emission from 400 to 650 nm.

### **CD spectroscopy**

CD spectra were recorded on a Jasco J-1500 CD spectrophotometer (Jasco Inc., Tokyo, Japan) between 250 and 185 nm at 20 °C, using a 1 mm quartz cuvette. The experimental parameters were as follows: band width, 1 nm; data pitch, 0.5 nm; scanning speed, 50 nm/min, response D.I.T. 1 s; accumulations, 5; the CD spectrum of the blank was subtracted from each of the recorded spectra.

CD spectra analysis was performed using DICHROWEB software, allowing to determine the fractions of secondary structure elements on the protein (Whitmore & Wallace, 2008), before

and after thermos-electric treatments. The analysis program used was CONTIN with the reference set 6, optimized for the wavelength interval of 185-240 nm.

### **Determination of Accessible Sulfhydryl Groups**

Determination of the free sulfhydryl groups (SH) was performed using Ellman's DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) method (Ellman, Courtney, Andres, & Featherstone, 1961) with some modifications. Briefly, 5 mM solution of DTNB was prepared in phosphate buffer, 0.1 M, pH 8 and stored at 4 °C, protected from light. On a glass test tube 2.5 mL of phosphate buffer (0.1 M, pH 8), 0.5 mL of protein solution and 100 µL of DTNB solution were mixed and allowed to react for 1 h at room temperature. The absorbance at 412 nm was determined on a UV-VIS spectrophotometer (V-560, Jasco Inc., Tokyo, Japan). All determinations were performed in triplicate and the absorbance of the blank (using buffer instead of protein solution) were subtracted on each sample absorbance. The total amount of thiol groups was determined using a positive control produced as described above, but using an 8 M urea solution at pH 8 instead of the phosphate buffer. The results were expressed as percentage of the total amount of free SH.

All chemicals were of analytic grade and purchased from Sigma-Aldrich (Steinheim, Germany). Double distilled water was used for the preparation of all solutions.

### **Statistical analyses**

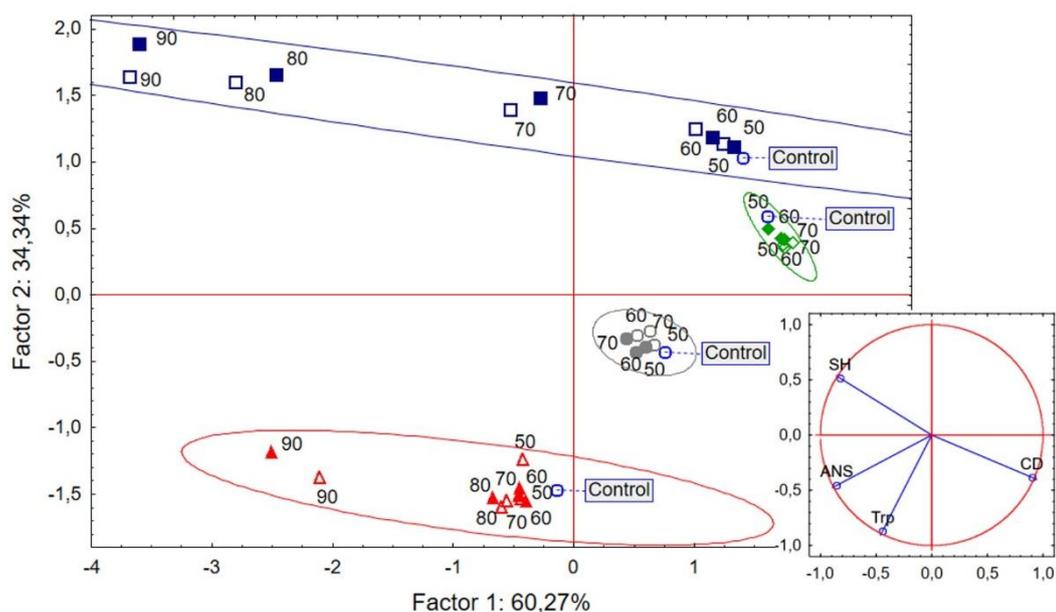
The statistical analyses of the experimental data were executed using Statistica package software version 10 (StatSoft Inc., Tulsa, OK, USA) applying an analysis of variance to estimate any statistically significant differences at a confidence level of 95%. Unless otherwise stated, all experiments were run at least in triplicate. For the Principal Component Analysis (PCA) the average values of the determined variables (i.e. maximum fluorescence intensity for both Trp and ANS, percentage of the SH groups and the ellipticity at 208 from DC) were used after being normalized and scaled.

## Results and Discussion

### Effects of physicochemical conditions

The aim of this work was to determine the influence of MEF on a wide-ranging spectrum of conditions. In this sense, Cov and OH treatments were performed in a series of temperatures and pH conditions, establishing the treatment type as the only variable of analysis. Nonetheless, the temperatures of 80 and 90 °C for the pH values of 4.3 and 5.7 were not considered on the analysis. Under these conditions, the treated samples presented high turbidity and in some cases, the protein precipitated, indicating excessive and uncontrolled aggregation. These insoluble aggregates are not suited for the analytic methods used in this work and do not fulfilled the requirement of the intended study of structural features. The appearance of these insoluble aggregates is explained by the proximity of the isoelectric point of the protein, and therefore the low charge and the consequent low repulsion occurring in these conditions. This, along with exposure to temperatures above denaturation, caused the reported aggregation even at such low protein concentration (Chi, Krishnan, Randolph, & Carpenter, 2003; Majhi et al., 2006). In all the other treatment conditions, the solutions remained clear and the absence of substantial aggregation was verified by the dynamic light scattering technique (results not shown).

Variations on the CD spectra, Trp and ANS fluorescence intensities and exposure of SH groups can be used to evaluate changes in conformation and structure of proteins. Using these data, a PCA was performed to determine the overall behaviour of the samples under the treatments applied. Figure 1 shows the PCA of the determinations performed and the corresponding projection of each treatment point. According to the analysis, the two main principal components, the first (horizontal axis) and second (vertical axis), accounted for 94.61 % of the variability found in measured data, showing that the analysis by a bivariate dimension is satisfactory.



**Figure 1:** Principal component analysis of measurements performed in ohmic and conventionally treated samples. The colours red, grey, green and blue represent the samples at pH 3, 4.3, 5.7 and 7 respectively and the ellipses represent a 95% confidence interval for each pH group. The open symbols represent conventional treatments and the closed ones represent OH. The corresponding treatment temperatures are placed next to the respective symbol. Figure insert shows the projection and correlations between variables measured.

The samples dispersion resulted in four groups correspond to the intervals of the four pH values tested, each of them represented by the 95 % confidence ellipses. The differentiation of these groups by the vertical factor is caused by the pH variation and the factor value is zero near the isoelectric point of the protein (i.e. 5,1). The vertical separation of the samples is more influenced by the fluorescence determination, suggesting the pH effect to be related with the conformation of the protein (i.e. hydrophobic sites accessibility and Trp exposure and micro-environment) and not with profound structural changes, that could be determined by SH groups exposure and significant changes in the secondary structure. On the other hand, the distribution along the horizontal axis is caused by the temperature increase and reflects structural change. This is the result of irreversible conformational changes occurring once the denaturation temperature was overcome.

For pH 3, despite a general tendency of the treated samples to be located to the left of the unheated sample, only the samples treated at 90 °C present a clear differentiation. Under these pH conditions,  $\beta$ -lg thermal stability is increased (Kella & Kinsella, 1988) and structural changes were only appreciable at the highest temperature tested. At pH of 4.3, there is few differentiation of the samples, although a tendency for small conformational changes (as the samples moved left and up from the unheated control) is apparent for increasing treatment temperatures. In contrast, at pH 5.7 the tendency is the opposite, as the samples move to the right and down. For pH 7 the clearest tendencies and higher differentiation between samples are found. A strong displacement to the left and along the vertical axis is caused by the increase of treatment temperature ( $\geq 70$  °C). This indicates that at neutral pH  $\beta$ -lg is more susceptible to thermally-induced structural changes. In any case, whenever the thermal treatment induces higher structural changes, the samples heated under OH show a clear differentiation from the Cov.

With the clear differentiation of the samples into four groups reflecting the environment pH, the subsequent analysis of the results and MEF effects determination was carried for each group individually and comparing only Cov and OH treatments. For the further discussion and aiming at focussing in the MEF effects on  $\beta$ -lg unfolding, only the treatments that presented statistic differentiation from the unheated samples and between treatment types will be considered, namely pH 3 90 °C and pH 7, 70 °C, 80 °C and 90 °C . The complete data set containing the average values, standard deviation and statistical significance analysis, for all the treatment performed is presented as supplementary material.

### **MEF effects in conformation and structure of unfolded $\beta$ -lg**

#### **Secondary structure**

CD spectra analysis presented in table 1 shows that unheated samples, corresponding to the native conformation of the proteins, present small differences in secondary structure fractions

according to the environmental pH. At pH 7  $\beta$ -sheet is slightly higher than at pH 3, where  $\alpha$ -helix is favoured.

**Table 1:** Comparison of the percentage of secondary structures of  $\beta$ -lg from CD spectra analysis.

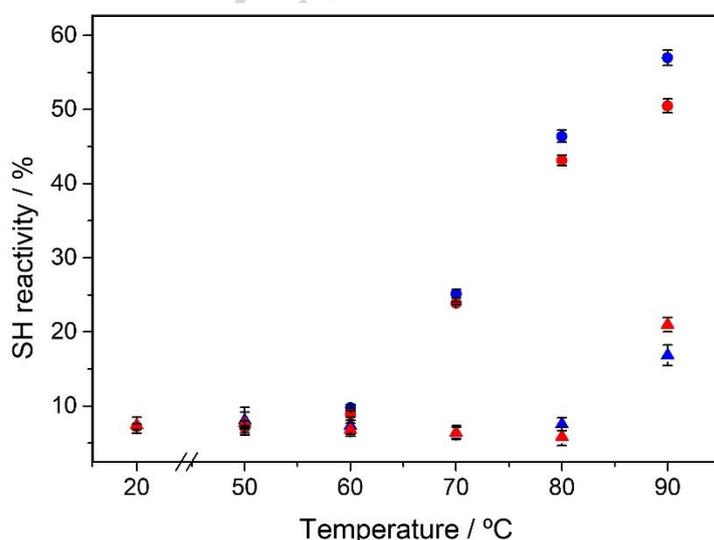
pH	Sample	$\alpha$ -helix	$\beta$ -sheet	Turn	coil
pH 7	Unheated	13.0 $\pm$ 0.7	42.0 $\pm$ 0.7	22.4 $\pm$ 0.6	22.6 $\pm$ 0.9
	Cov 70	14.6 $\pm$ 0.9	34.8 $\pm$ 0.5	22.5 $\pm$ 1.0	28.1 $\pm$ 0.6
	OH 70	15.4 $\pm$ 0.5	31.4 $\pm$ 0.8	23.7 $\pm$ 0.6	29.5 $\pm$ 1.6
	Cov 80	16.4 $\pm$ 0.9	26.3 $\pm$ 0.6	21.2 $\pm$ 0.9	36.2 $\pm$ 0.4
	OH 80	20.8 $\pm$ 0.8	20.4 $\pm$ 1.0	19.7 $\pm$ 0.5	39.1 $\pm$ 0.8
	Cov 90	20.5 $\pm$ 0.8	21.0 $\pm$ 0.7	21.3 $\pm$ 1.2	37.2 $\pm$ 0.3
	OH 90	16.2 $\pm$ 0.4	24.4 $\pm$ 0.5	23.0 $\pm$ 0.8	36.5 $\pm$ 1.1
pH 3	Unheated	16.8 $\pm$ 0.3	40.7 $\pm$ 0.5	22.4 $\pm$ 1.3	20.1 $\pm$ 1.7
	Cov 90	20.1 $\pm$ 0.6	31.3 $\pm$ 1.5	21.5 $\pm$ 0.6	27.1 $\pm$ 1.5
	OH 90	19.2 $\pm$ 0.8	35.1 $\pm$ 1.1	23.3 $\pm$ 1.0	22.5 $\pm$ 1.6

After thermal treatments it was verified changes in the structure of  $\beta$ -lg attributed to the loss of secondary structural elements. These changes potentiated by the temperature increase, were characterized by a progressive loss of  $\beta$ -sheet, a slight increase in helical content and increase of random coil. Nonetheless, the overall loss secondary structural elements is not radical and substantial fractions of  $\beta$ -sheet are preserved and  $\alpha$ -helix content increases. This may indicate that the main structural features of  $\beta$ -Lg, namely the central barrel, is preserved at least partially, resulting on a non-native forms known as molten globule (Bhattacharyya & Varadarajan, 2013; Croguennec, Mollé, Mehra, & Bouhallab, 2004). At pH 7 the secondary structural changes are more extensive and start at 70 °C. Samples treated by OH present different structural features than the conventional ones, presenting lower content of  $\beta$ -sheet and higher helical and random coil in 70 °C and 80 °C treatments. For treatments at 90 °C the behaviour inverts and OH treated samples display higher content of  $\beta$ -sheet helix and random coil than the Cov. Interestingly samples treated by OH at 80 °C, present secondary structural features similar to the

conventional treatment at 90 °C, being the most distant from the native conformation, reaching changes up to 22 % in  $\beta$ -sheet loss, 7 % increase of helical content and 17 % in random coil. At pH 3, a similar behaviour was found, nonetheless with lower changes in magnitude, as treatments at 90 °C result in a distribution of secondary structural elements similar to the treatments at pH 7 and 70 °C. Again, OH at 90 °C results in higher  $\beta$ -sheet and lower helical and random coil content.

### Free SH group relativity

SH reactivity is not only dependent on the accessibility of cystein (Cys) 121 but also on the disulphide bond brake and disulphide interchange, usually occurring above 80 °C, resulting on the reactivity of other Cys residues with different accessibility (Halder, Chakraborty, Das, & Bose, 2012). In figure 2 is presented the SH reactivity for all the treatments at pH 3 and 7. Unheated samples, presents on the 20 °C mark, exhibit similar SH reactivity in both pH values. The higher reactivity of SH groups at pH 7 is favoured by the higher structural changes observer and for the increased access to the central barrel known as the Tanford transition. SH reactivity increased significantly above 60 °C for treatments at pH 7, supporting literature reports which confirm this temperature as starting point to make Cys 121 accessible (de Wit, 2009; QI et al., 1997). With higher treatment temperatures, also increases the amount of free thiols, reaching more than 50% and OH treated samples consistently presented a lower amount of reactive SH.

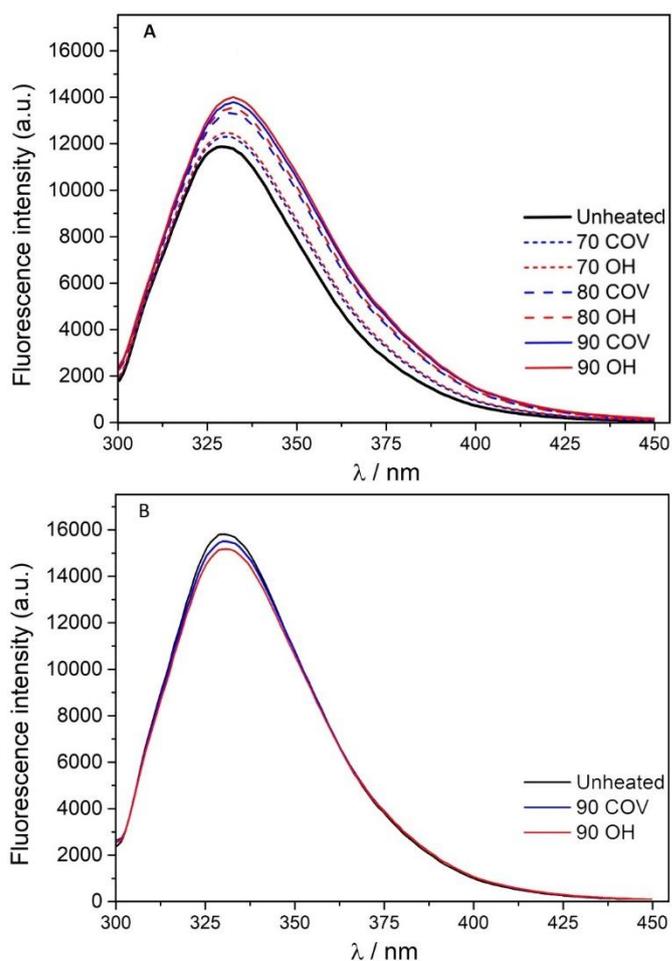


**Figure 2:** Reactivity of SH for treatments at pH 3 (triangles) and pH 7 (circles). Red markers represent OH treatments and the blue ones the Cov treatments.

At pH 3, along with all the other determinations, SH reactivity increases only for treatments at 90 °C, contrasting with much earlier response at pH 7. Also the SH reactivity reached fractions of 21 % and 17 % for Cov and OH treatments respectively, smaller than the observed in the treatments at neutral pH, which reached about 25 % already at 70 °C treatments. Interestingly, samples treated at 90 °C by OH present higher SH reactivity than the equivalent Cov treatments. SH reactivity correlates with the structural changes happening upon thermal stress, increasing as the structural changes take place. However, the presence of MEF seems to promote different actions in pH 3 and 7. The favouring or restriction of SH reactivity imposed by MEF on the reported conditions can be resultant on the different conformations imposed by the pH conditions and inherent changes in charge distribution, which should be able to promote rearrangements in structural elements or even influence intramolecular disulphide exchanges.

### **Intrinsic fluorescence**

$\beta$ -lg's fluorescence is attributed mainly to the Trp 19 residue, placed deep inside the hydrophobic barrel (Albani, Vogelaer, Bretesche, & Kmiecik, 2014) while the Trp 61 is mostly quenched by the nearby Cys66-Cys160 bond (Croguennec et al., 2004). Therefore, variations in Trp fluorescence intensity and shifts in fluorescence peak and spectra shape can contribute with valuable information about protein conformation and intramolecular changes. In figure 3 are presented illustrative fluorescence spectra of Trp in  $\beta$ -lg under different conditions.



**Figure 3:** Trp fluorescence spectra of native and treated  $\beta$ -Ig samples at pH 7 (A) and pH 3 (B). Excitation wavelength was 295 nm.

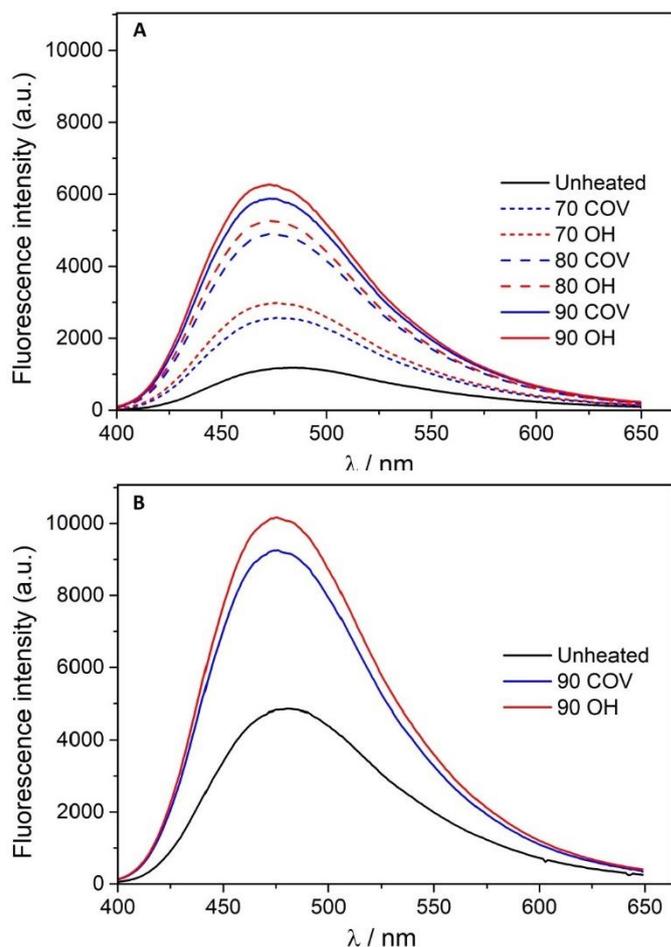
At pH 7 the unfolded proteins shows an increase in Trp fluorescence, as well as a red shift ( $\leq 5$  nm) in the maximum fluorescence and an elongation of the spectra to the right, indicating a change from an apolar to a more polar Trp microenvironment (Halder et al., 2012; Sahihi, Ghayeb, & Bordbar, 2012). The OH samples from 70 to 90 °C present small but significantly higher intensities than Cov, indicating changes in Trp exposure or quenchers accessibility. At pH 3, the Trp fluorescence is considerably higher than the observed at pH 7 which may be caused by the lower accessibility of Trp 19, once in acid environments  $\beta$ -Ig presents a closed conformation and displacement of some structural element's orientation as the long  $\alpha$ -helix (Uhrínová et al., 2000). In these conditions, Trp fluorescence suffered few changes, decreasing the maximum intensity for 90 °C treatments and presenting a smaller red shift of 2 nm; again

OH treated samples present higher changes than the Cov treated samples. This decrease in Trp fluorescence intensity is opposite to the observed at pH 7, also the red shift observed is smaller in the treatments at different pH values. This may be correlated with the reactivity of SH and disulphide bonds distribution. The interchange of any of the two disulphide bonds among them or with Cys 121 would result on different quenching equilibrium of the two Trp residues. An interchange in Cys66-Cys160, leaving any of these Cys free would cause a reduced quenching in Trp 61, resulting on a higher contribution of this residue, increasing the fluorescence intensity and also the red shift. On another hand, an exchange in Cys106-Cys119 bond with Cys 121 would position de disulphide bond closer to the Trp 19, causing an increased quenching and reduce the fluorescence intensity. As this Trp is located inside the  $\beta$ -barrel the microenvironment is not expected to suffer substantial changes and thus lower shift would be expected. Several other mechanism may take place and probably all of them occur simultaneously in molecules of the same sample, yet with the prevalence of some mechanisms against others. Nonetheless, the two scenarios described are consistent with the observations reported, where after thermos-electric treatments at pH 7 the Trp fluorescence increases with a larger red shift than at pH 3 where the Trp fluorescence diminishes.

In both pH values, the presence of MEF induced higher changes in Trp fluorescence intensities; however, no apparent shift on the maximum intensity or distortion of the spectra were observed between treatment types. This suggests that no differences in Trp positioning were induced by the presence of MEF.

### **ANS binding**

ANS fluorescence intensities suffer major changes depending on the pH value. These modifications relate with conformational changes induced by protonation/deprotonation of some groups and overall surface charge of the protein (Sakurai, Konuma, Yagi, & Goto, 2009; Taulier & Chalikian, 2001). It is thought that the main binding site of ANS to  $\beta$ -lg is on the opening of the central hydrophobic barrel, but at low pH a second binding site at the surface has been also reported (Collini et al., 2003; Collini, D'Alfonso, & Baldini, 2000).



**Figure 4:** ANS fluorescence spectra of native and treated  $\beta$ -lg samples at pH 7 (A) and pH 3 (B). Excitation wavelength was 370 nm.

Figure 4 shows ANS fluorescence spectra of untreated samples and samples treated at 90 °C for pH 3 and at 70, 80 and 90 °C for pH 7. The higher ANS fluorescence at pH 3 results from the availability of the external binding site, that is not available at higher pH values. At pH 7, ANS fluorescence suffers the largest relative increase (related to the unheated sample at the same pH) with increasing treatment temperatures. This is in agreement with the secondary structure analysis that suggests a partial unfolding with the conservations of substantial fractions of the  $\beta$ -barrel, resulting on an enhancement of the access to the barrel. For all the OH treated samples, regardless of the environmental pH value, ANS fluorescence is higher than the correspondent Cov treatment, which demonstrates that MEF action increased hydrophobic groups accessibility. The capability of MEF to affect hydrophobic and SH groups disposition implies important

consequences in functionality, as the interactions established by these groups define protein aggregation and association with other compounds as drugs or bioactives (Mudgal, Daubert, & Foegeding, 2011; Vetri & Militello, 2005).

### **MEF mechanism in protein unfolding**

The effects of MEF in proteins have been reported previously and demonstrated to change structure and activity of proteins, either by applying MEF at room temperature (without thermal effects) and with long exposure or on enzyme activity associates with a specific temperatures of activation and deactivation (Bekard & Dunstan, 2014; Samaranayake & Sastry, 2016b, 2016c, 2018). It was proposed that the effects of an external electric field are related with molecular motions induced from oscillatory behaviour of the EF and the consequent energy dissipation through frictional drag. In fact Samaranayake & Sastry (Samaranayake & Sastry, 2016a) propose that the action of MEF is related with the increase energy dissipation and consistent with an apparent elevation of temperature on the protein molecules. The level of energy involved in these motions was estimated to be equivalent or greater to the hydrogen bonds that stabilize the protein fold and thus they are susceptible of being destabilize or disrupted. These effects are more effective at low frequencies, where the oscillatory motions have greater amplitude or even induce full rotation. Higher frequencies, as the ones used in this study, induce vibrational motion, more chaotic with unstable and aperiodic motion (Kouzaev, 2013). The conditions used in this study involved low exposition time to MEF and the use of high frequencies, which must have limited the effectiveness of electric effects, thus explaining the absence of effects at low temperature treatments. However, our results demonstrate that, the presence of MEF during OH influenced the structural features in non-native forms of  $\beta$ -Lg. For higher treatment temperatures, the increased entropy imposed and the disruption of the protein native fold may allow the vibrational effects and additional energy dissipation to effectively induce changes in conformation upon rearrange the structural elements.

The proposed mechanism of MEF action is supported by the experimental data where the differentiation of OH and Cov treated samples was only evident in conditions where substantial structural changes were induced by thermal action. Furthermore, the conformational rearrangements imposed by the environmental pH affects the specific action of MEF. Upon thermal unfolding the MEF presence resulted in significant changes in secondary structural elements, Trp fluorescence, hydrophobic sites accessibility, SH reactivity and possibly different disulphide rearrangement.. These facts are consistent with an additional energy dissipation resultant from the molecular motion induced by the electric field. None of the reported MEF effects represents a disruptive behaviour when compared to the Cov treatments, but results show an evidence of local conformational changes that should not be overlooked once can impact functional and technological properties of  $\beta$ -lg. In this sense, the understanding of the specific effects and mechanisms involved in MEF action are fundamental to control protein functionality -i.e. complex formation or self-aggregation in nanostructures and gel systems- and further investigation should be carried out to better understand these mechanisms in  $\beta$ -lg, and proteins in general.

## Conclusions

MEF processing is a recognized emerging technology that can be used as a tool to change protein physicochemical properties, allowing to obtain differentiated products with distinctive features. In this work, the effects of MEF on the unfolding of  $\beta$ -lg have been demonstrated through structural and conformational characterization by differentiation in the far-UV CD spectra, reactivity of SH groups, Trp and ANS fluorescence. Despite the previously reported effects of MEF in protein structure, this is the first time that MEF influence was verified at molecular level in thermal processing treatments. Our results have shown that MEF effects are limited to unfolded forms of the protein, suggesting that in the conditions used (i.e. electric field strength, frequency and time of exposure) the disturbances imposed are not sufficient to disrupt the protein fold and MEF effects are therefore synergic with the thermal effects. Furthermore,

their specific action and extent was dependent of pH, which may be a result of the dynamic  $\beta$ -Ig conformation in response to the environmental conditions. These results corroborated the hypothesis postulated in previous works where MEF effects were proven to influence aggregation and gelation of whey proteins. These findings contribute also to a more comprehensive understanding of the pathways involved in the dynamic behaviour of this protein and to define MEF as technological tool to control protein functionality and unravel potential interactions with other bioactive molecules.

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## Industrial relevance

Ohmic heating is an emerging technology and is being established as reference method for processing protein-rich food such as dairy and egg products. Non-thermal effects of the applied electric fields during ohmic heating have been addressed but few works deal with their real impact in structural and molecular properties of food proteins with high biological value.

In this work demonstrated that the presence of an electric field during ohmic heating processing influences structural aspects of beta-lactoglobulin. This knowledge plays an important role on process design (i.e. pasteurization binomials and fouling control) and product quality because these proteins play an essential role in food's nutritional and organoleptic properties, as well as on functionality, allergenicity and stability aspects.

## Highlights

- The effects of ohmic heating on the unfolding of  $\beta$ -lg was evaluated from 50 to 90 °C and pH 3 to 7
- The presence of moderate electric fields during ohmic heating have demonstrated to affect  $\beta$ -lg's conformation upon unfolding.
- MEF action results in different structural features, hydrophobic and sulfhydryl reactivity profiles, affecting protein functionality
- $\beta$ -lg's response to thermal and electric effects is dependent of the pH and is more susceptible to conformational and structural changes at neutral pH