LWT - Food Science and Technology 116 (2019) 108550

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Camel α−lactalbumin at the oil−water interface: Effect of pH and heat treatment on the structure, surface characteristics and emulsifying properties

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ARTICLE INFO

Keywords:
Camel milk
Emulsion
Alpha-lactalbumin
Hydrophobicity
Surface pressure

ABSTRACT

The effects of pH (3.0, 6.0, 9.0) and heat treatment (25 °C, 65 °C, 95 °C) on the emulsifying properties of α−lactalbumin extracted from camel milk were investigated and compared to bovine α−lactalbumin. The results show that both studied proteins displayed a rather different evolution of their emulsifying activity as a function of the main factors (pH and temperature); however, they exhibited very close maximum values, between 100 and 110 $m^2 g^{-1}$. The stability of camel α−lactalbumin stabilised emulsions at pH 3.0 and 9.0 was attributed to the associated electrostatic forces (ζ−potential ~ −30 mV). The emulsifying properties of camel α−lactalbumin were less sensitive to heat treatment (95 °C), due to the higher conformational flexibility, as surface hydrophobicity increased with temperature (from 16 A.U to 51 A.U); conversely, bovine α−lactalbumin enhanced emulsion stability vs. pH and heat treatment, due to hydrophobic interactions and a more rigid molecular structure. Statistical tests also showed that the adsorption of camel α−lactalbumin at the oil−water interface was significantly affected by pH change.

1. Introduction

Milk proteins are commonly used as surface active molecules, in particular whey proteins due to their nutritional importance and their wide range of functionality in food and pharmaceutical products including thickening, gelling, foaming and emulsification. The ability of milk proteins to adsorb at the oil−water interface and to stabilise emulsions is a very important functionality which has been exploited by many industries of food (cream liqueurs and dairy desserts), cosmetics (cleansers, body lotions) as well as of pharmaceutical products (encapsulation of active ingredients).

Camel milk (*Camelus dromedarius*) is considered as the main dairy product in certain regions of the world, particularly in Africa and Asia, and is well known for its exceptional therapeutic characteristics. First, studies involving camel milk mainly focused on the differences in composition with cow's milk. More recently, some functional properties of camel milk were explored, in particular their foaming activity (Hu, Ting, Hu, & Hsieh, 2017; Lajnaf et al., 2018; Lajnaf, Picart-Palmade, Attia, Marchesseau, & Ayadi, 2017a). A recent study using camel whey proteins in a model beverage emulsion (Momen, Salami, Alavi, Emam-Djomeh, & Moosavi-Movahedi, 2019) also showed their significant emulsifying properties compared to bovine whey in specific ranges of pH (3.3) and temperature (85 °C). Understanding the mechanism of camel milk protein adsorption at the oil−water interface at larger ranges of pH and temperature is, therefore, of great interest, especially for the food and the pharmaceutical industries.

Camel milk is marked by the absence of β−lactoglobulin (β−Lg) compared to cow milk; α−lactalbumin is thus known to be the most abundant whey protein of camel milk, exceeding 40% of total whey protein content (Lajnaf et al., 2017a). Camel α−lactalbumin (Ala−C) is a globular protein with a molecular mass of 14.6 kDa and an isoelectric point (pI) of 5.1–5.3 (Conti, Godovac Zimmermann, Napolitano, & Liberatori, 1985). Ala−C consists of 123 amino acid residues forming a compact globular structure stabilised by four disulphide bonds; Ala−C exhibits a high affinity for metal ions, calcium in particular. Sequence similarity and identity between camel and bovine α−lactalbumin are estimated at 82.9% and 69.1%, respectively (Atri et al., 2010). However, heating of Ala−C results in very little protein polymerization/aggregation due to the lack of free thiol groups in comparison to bovine α−lactalbumin (Ala−B) (Lajnaf, Picart-Palmade, Attia, Marchesseau, & Ayadi, 2017b). Structurally, α−lactalbumin is an amphiphilic protein elected to be a good emulsifier agent

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https://doi.org/10.1016/j.lwt.2019.108550
Received 13 May 2019; Received in revised form 13 August 2019; Accepted 25 August 2019
Available online 28 August 2019
0023-6438/

to produce protein-stabilised emulsions (Lam & Nickerson, 2013). When added to oil − water interface, the bovine α − lactalbumin proteins form a cohesive interfacial protein layer around oil droplets, providing steric and either electrostatic repulsive or attractive forces between emulsion droplets. The relative stability of emulsions against coalescence is governed by the balance between these forces. Major advances have been made to better understand the adsorption process of cow milk proteins at the oil − water interface, the structure and the composition of the adsorbed layer, and the influence of the physical and chemical environment on their emulsifying properties (Dickinson, 2001; Singh, 2011).

In this respect, understanding the emulsifying properties of camel's milk α − lactalbumin protein under different treatments is of great industrial and scientific interest promoting novel and interesting substitute to existing emulsifier. The main objective of this work is, therefore, to study the behaviour of camel milk α − lactalbumin protein (Ala − C) at the oil − water interface in a wide range of heat treatment temperature and pH, and to compare it to cow's α − lactalbumin protein in a low-fat emulsion model.

2. Materials and methods

2.1. Materials

Camel milk used in this study was purchased from a local farm in the region of Tozeur in south Tunisia. Just after milking, 0.2 g/L of sodium azide (NaN_3) was added to stop bacterial proliferation; then, milk was stored at 4 °C.

Cow's milk α − lactalbumin (Ala − B) was purchased from Sigma–Aldrich (USP Reference Standard). Protein content was measured to be 898.8 g/kg and this protein was used without further purification.

Local produced rapeseed oil for alimentary use was purchased from a local supplier and used without further purification. Water was produced using a Millipore Milli − Q™ water purification system (Millipore Corp., Milford, MA, USA). All other chemicals used in this study are of reagent grade and purchased from Sigma–Aldrich (USA).

2.2. Camel α − lactalbumin purification

Once the camel milk was skimmed by centrifugation (3800 × *g*, 20 min, 7 °C), milk casein was removed by acidic aggregation at pH 4.2 using hydrochloric acid (HCl, 1 mol/L), followed by centrifugation at 5000 × *g* for 15 min (Lajnaf et al., 2018). Supernatant purification was then carried out using a 50 kDa ultrafiltration membrane (VivaFlow 200, Sigma–Aldrich, USA). A pH adjusted water at 6.7 was used to refilter the retentate of the purification. The washing process was carried out six times. Between each cycle, the initial flow of ultrafiltration membrane was restored using a concentrated NaOH solution (10 mol/L) and rinsed with Milli − Q™ water. Once the permeate was collected, it was concentrated using a 5 kDa ultrafiltration membrane (VivaFlow 200, Sigma–Aldrich, USA), and then dialysed against Milli − Q™ water at 4 °C and steady stirring. Water was replaced every 12 h for 4 days (Salami et al., 2009). The protein content was 760 ± 2 g/kg, measured using a total nitrogen analyser (TNM − 1, Shimadzu Corp., Japan) and a nitrogen conversion factor of 6.38. A protein profile (sodium dodecyl sulphate polyacrylamide gel (12% SDS − PAGE)) was achieved at each step of the purification process to test the Ala − C purity. The purified protein was freeze − dried and stored at 20 °C for further usage.

2.3. Sample and emulsion preparation

The α − lactalbumin stock solutions (2 g/L, corrected for protein content) were prepared by dispersing Ala − C and Ala − B lyophilised powders in Milli − Q™ water. pH was adjusted to 3.0, 6.0 and 9.0 using either 0.5 mol/L HCl or 0.5 mol/L NaOH, followed by mechanical stirring (550 rpm) at room temperature (23–25 °C) for 90 min. Protein solutions were then poured into screw − capped 15 mL centrifuge tubes to prevent moisture losses and held in a water bath for 15 min at 25 °C (room temperature), 65 °C and 95 °C. Tubes were, then, cooled down in an ice bath to room temperature prior to further analysis.

Finally, emulsions were prepared by mixing 5 g of the α − lactalbumin stock solution with 20% (w/w) of rapeseed oil within a 50 mL plastic centrifuge tube, followed by homogenisation at 21,500 rpm for 3 min using an Ultraturrax T25 homogenizer (Ika − Werke GmbH, Germany) equipped with a SN25 − 10G ST tool.

2.4. Emulsion properties

Emulsion stability and activity indexes (ESI and EAI, respectively) were measured according to the modified method of Lam and Nickerson (2015a). Immediately after homogenisation, aliquots of 50 μL were transferred into 10 mL of a pH − adjusted aqueous solution containing 1 g/kg sodium dodecyl sulphate (SDS) and vortexed for 10 s. Optical density was read at 500 nm using an ultraviolet–visible spectrophotometer (Biomate 2S, ThermoFisher Sci., USA) and plastic cuvettes (1 cm path length). After 10 min, a second aliquot of the same emulsion was taken, following the previous procedure. The EAI and ESI were then calculated using Eqs. (1) and (2), respectively:

$$\text{EAI}\ (\text{m}^2.\ \text{g}^{-1}) = \frac{2 \times 2.203 \times N \cdot A_0}{10^5 \times C \cdot \phi} \quad (1)$$

$$\text{ESI}\ (\text{min}) = \frac{\text{A}_0}{\Delta \text{A}}.\text{t} \quad (2)$$

where A_0 is the absorbance of the diluted emulsion immediately after homogenisation, N the dilution factor (250), C the weight of protein per volume (g/ml), ϕ the oil volume fraction in the emulsion, ΔA the difference of the absorbance between at time 0 and time 10 min (A_0 - A_{10}), and t the time interval (10 min).

2.5. Emulsion ζ–potential

ζ–potential is a key property of emulsions (McClements, 2006), was measured using a Zetasizer Delsa Nano HC (Beckman Coulter, USA). The emulsion samples were diluted 1:100 (v/v) using Milli − Q™ water. Samples were equilibrated for 120 s before collecting data. The sampling time was fixed at 400 μs. Data were accumulated from 10 sequential readings at 25 °C, and the mathematical model of Smoluchowski (Sze, Erickson, Ren, & Li, 2003) was selected to convert the electrophoretic mobility measurements into ζ–potential values using Eq. (3):

$$\zeta = \frac{3}{2}\frac{\eta}{\varepsilon}U \quad (3)$$

where ζ is the measured ζ–potential (mV), η the viscosity of the emulsion (Pa.s), ε the permittivity of water, and U the electrophoretic mobility (m^2/V.s).

2.6. Droplet size and microscopic observation

The droplet size distribution was determined using a laser scattering technique (Mastersizer 3000E, Malvern PANAnalytical, UK). Just after homogenisation, 1 ml aliquot of each emulsion was gently blended to an equal volume of pH − adjusted water containing 10 g/L sodium dodecyl sulphate (SDS) to avoid multi − scattering effect and prevent emulsion flocculation. Emulsion droplet sizes were measured under steady agitation (1500 rpm) and reported as the Sauter mean diameter, d_{32}, defined as:

$$d_{32} = \sum n_i d_i^3 \Big/ \sum n_i d_i^2 \quad (4)$$

where n_i is the number of particles of diameter d_i.

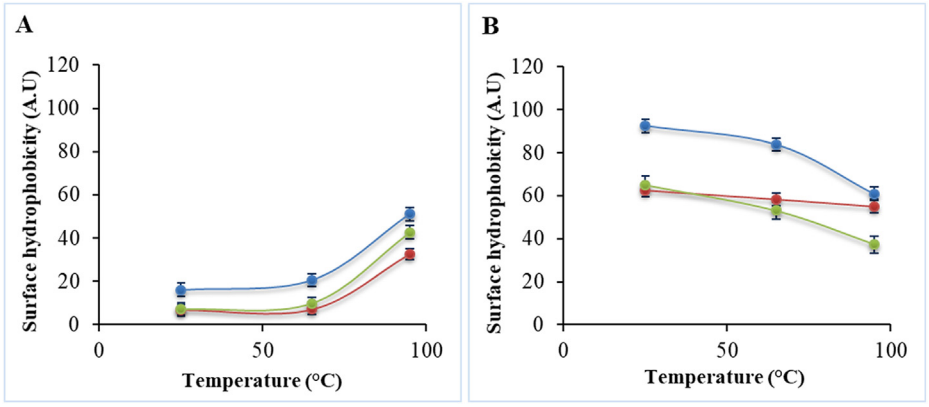

Fig. 1. Surface hydrophobicity (A.U.) of Ala−C (A) and Ala−B (B) proteins in terms of temperature of heat treatment for different pH (●) 3.0, (●) 6.0, (●) 9.0. Data represent the mean ± standard deviation (n = 3).

Microscopic observations were carried out using an Axiovert 25 inverted microscope (Carl Zeiss GmbH, Germany) equipped with a monochrome Pulnix camera (JAI, Japan, 640 × 480 pixels). Emulsion aliquots of 20 µL were placed onto a microscope slide and carefully covered with a coverslip, avoiding any bubble formation. Micrographs were recorded at × 100 magnification.

2.7. Surface hydrophobicity and interfacial tension

Surface hydrophobicity of Ala−C and Ala−B solutions treated under different conditions of pH and temperature were measured according to the modified method of Alizadeh-Pasdar and Li-Chan (2000) using a spectrofluorimeter Flx (SAFAS, Monaco). An 8−anilino−1−-naphthalenesulfonate (ANS) probe was added to interact with hydrophobic moieties on the protein surface to give a fluorescent signal. Each protein solution was diluted to a concentration of 0.8 g/L. 20 µL of ANS (8.10^{-3} mol/L) solution dissolved in a phosphate buffer (50.10^{-3} mol/L, pH 7.0) was added to 4 mL of each protein solution. The solution was excited at 390 nm, and the emission spectrum was measured from 400 to 600 nm. The emission and excitation slits were set to 5 nm, and the measurements were performed at 25 °C. The maximum area of the fluorescence spectrum was corrected with the buffer area. Surface hydrophobicity is reported as the maximum of the obtained spectrum.

The interfacial tension for each protein solution was measured using a K12 tensiometer (Krüss GmbH, Germany) equipped with a platinum Wilhelmy's plate to achieve complete wetting (contact angle θ is 0, *i.e.* $\cos(\theta) = 1$). Within a 20 mm diameter glass sample cup, 5 mL of protein solution (1 g/L of protein content) was added, followed by the immersion of Wilhelmy's plate; then, an upper layer of rapeseed oil (10 mL) was poured over it. The measurement time was fixed to 2000 s. The interfacial tension was obtained by correlating the force F (mN) applied on the immersed plate to the wetted length of the plate L (mm) between the plate and the liquid as expressed by Eq. (5):

$$\sigma\left(\frac{\text{mN}}{\text{m}}\right) = \frac{F}{L}\cos(\theta) = \frac{F}{L} \quad (5)$$

The resulted data was displayed by the change in interfacial tension from the pure fluid value/log time (Eq. (6)), which gives access to an easy comparison between systems with different σ_0 (the interfacial tension of pure fluids), and helps visualize the diffusion rate of proteins at the oil/water interface (Beverung, Radke, & Blanch, 1999).

$$\Pi(t) = \sigma_0 - \sigma_t \quad (6)$$

where Π is the surface pressure, and σ_t the measured interfacial tension at time *t*.

2.8. Rheological behaviour

Rheological measurements were conducted at 25.0 °C using an AR−G2 rheometer (TA Instruments, USA) equipped with a 40 mm standard steel parallel plate. 2−ml aliquots of freshly prepared emulsions were used per measurement. An up and down shear rate sweep test was carried out between 0.1 s^{-1} to 1000 s^{-1} in order to measure emulsion viscosity. For all measurements, the gap distance was fixed at 1000 µm.

2.9. Statistics

All analyses were performed in triplicate and reported as the mean ± standard deviation. A two−way analysis of variance (ANOVA) was applied to test for significance of the main effects, *i.e.* pH (3.0, 6.0, 9.0) and heat treatment (25.0, 65.0, 95.0 °C), along with their associated interactions, on the physicochemical and emulsifying properties of Ala−C and Ala−B proteins. A quadratic model with a second−order interaction term was assumed to correlate by linear regression the physicochemical and emulsifying properties of proteins to the main factors, as expressed in Eq. (7):

$$Y_i = a_0 + a_1.pH + a_2.T + a_3.pH^2 + a_4.T^2 + a_5.pH.T \quad (7)$$

where Y_i is the tested response, a_i are the observed effects, and pH and T are the main factors. The significance of the effects is expressed in terms of the p−value (p) which tests the null hypothesis that the coefficient is equal to zero (no effect). Statistical analyses were carried out using IBM SPSS Statistics (Ver. 20, IBM, USA).

3. Results and discussion

3.1. Surface properties of camel and bovine α−lactalbumin

3.1.1. Surface hydrophobicity

Fig. 1 shows the evolution of surface hydrophobicity of Ala−C and Ala−B as a function of pH (3.0, 6.0 and 9.0) and heat treatment (25 °C, 65 °C and 95 °C). Globally, the surface hydrophobicity of Ala−C proteins is lower than Ala−B proteins at most treatments. Figure 1B illustrates that Ala−B protein's hydrophobicity decreases when the temperature of heat treatment increases at pH 3.0 and 9.0, while it remains nearly constant for pH 6.0. Contrary to Ala−B, surface hydrophobicity of Ala−C proteins (Fig. 1A) increases only when the temperature of heat treatment is high (95 °C) for all pH values. However, it must be pointed out that the effect of pH is the same for both proteins: surface hydrophobicity is significantly higher at pH 3.0, while at pH 6.0 and 9.0, differences emerge only after heat treatment above 65 °C, as described by ANOVA in Table 1. Ala−B proteins probably aggregate through hydrophobic interactions at these conditions (pH 3.0 and pH 9.0), resulting in a reduced hydrophobicity. The stable behaviour at pH 6.0 might be explained by its globular rigid conformation and its enhanced resistance to heat treatment close to pI (Lam & Nickerson, 2015b; Zhai et al., 2012). For camel milk proteins, this

Table 1
Statistical results from a 2-way ANOVA describing the physicochemical and emulsifying properties of Ala − C and Ala − B proteins as a function of pH and temperature treatments.

Factors		***pH***	***T***	***pH*²**	***T*²**	***pH* × *T***
EAI	Ala − C	***	NS	***	NS	NS
	Ala − B	***	NS	***	NS	***
ESI	Ala − C	*	NS	NS	NS	NS
	Ala − B	**	NS	**	NS	NS
ζ − Potential	Ala − C	**	NS	NS	NS	NS
	Ala − B	*	NS	NS	NS	NS
Surface hydrophobicity	Ala − C	**	***	*	***	*
	Ala − B	***	***	***	***	***
d_{32}	Ala − C	***	*	***	NS	**
	Ala − B	NS	**	**	***	NS
Surface pressure	Ala − C	***	NS	***	NS	NS
	Ala − B	NS	***	*	**	*
Viscosity	Ala − C	***	NS	***	NS	NS
	Ala − B	*	**	NS	**	NS

Note that NS means 'not significant', referring to a $p > 0.05$, (*) means $0.01 < p \leq 0.05$, (**) $0.001 < p \leq 0.01$, (***) $p \leq 0.001$.

evolution is explained by the exposure of hydrophobic moieties at high heat treatment, which highlights partial denaturation enhanced at acid pH (3.0). Several researchers had already investigated the denaturation and aggregation behaviour of bovine milk proteins. Irreversible denaturation was reported at a temperature above 90 °C. This is due to the susceptibility of cow whey protein, in particular Ala − B, to high heat treatment, as mentioned previously in several previous works (Elagamy, 2000; Laleye, Jobe, & Wasesa, 2008). These results show that the flexible molecular structure observed for Ala − C is affected by pH variation and heating temperature and leads to a conformational reorganisation to possibly align at the oil − water interface.

3.1.2. Interfacial properties

Interfacial tension expressed as surface pressure for Ala − C and Ala − B treated proteins is plotted in Fig. 2. For Ala − B proteins (Fig. 2B), the surface pressure reaches the highest value (1.7 mN/m) at alkaline pH, particularly after heat treatment at 65 °C. Ala − B treated proteins reduce less rapidly and less efficiently the interfacial tension between the two immiscible phases than Ala − C proteins; the induction time of Ala − B (i.e. the time from the start of the measurement until the first change of the curve slope) which reflects the first changes of protein's conformation to adsorb at the contact between the low and high density phases (oil and water, respectively) is estimated at 300 s. At higher pH, the decrease in interfacial tension is favoured by a simultaneous increase in temperature and pH, which is corroborated by the significant effect of the interaction terms ($pH \times T$) of the ANOVA table (Table 1). This is attributed to the ability of adsorbed proteins to change their conformation and unfold in response to hydrophilic/hydrophobic side chains and the enhanced interactions with the oil phase (Beverung et al., 1999; Miller et al., 2000). Contrary to Ala − B, the surface pressure of Ala − C proteins (Fig. 2A) reaches 2.1 mN/m at pH 3.0 mainly at low-temperature heat treatment. For most treatments, the induction time is estimated to 80 s for Ala-C. At pH 3.0 where the degree of denaturation was higher (95 °C), the second diffusion regime of proteins at the interface (which starts from the first change of curve slope until the second change, it reflects, therefore, the diffusion-controlled adsorption of the first layer of proteins at the interface) was observed instead. ANOVA (Table 1) shows a very significant effect of pH ($p < 0.001$). This suggests that the diffusional regime of the Ala − C proteins at oil − water interface is mainly governed by electrostatic forces between the charged moieties of both phases. Thus, unheated Ala − C proteins present an adequate molecular conformation to interact with the fatty acids of rapeseed oil, resulting in a very significant reduction in interfacial tension.

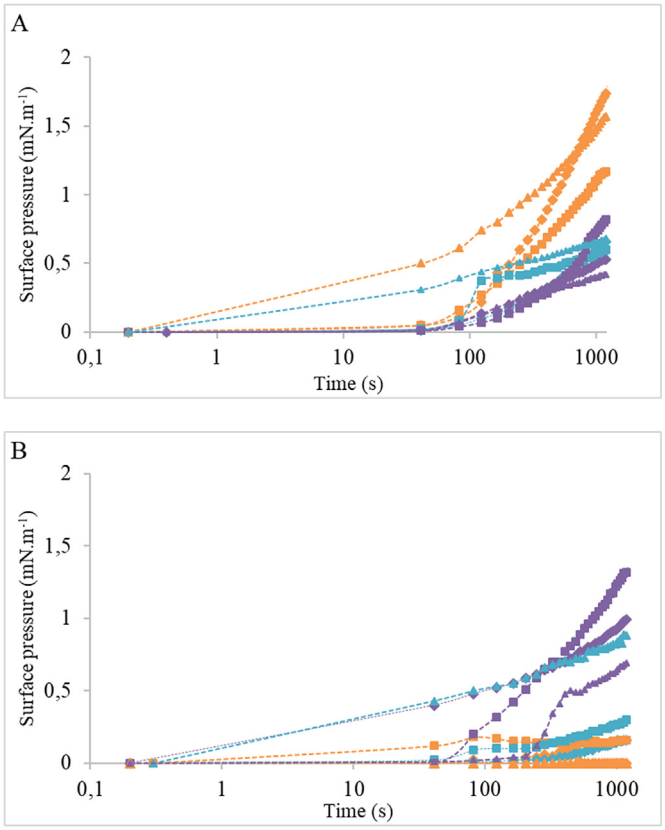

Fig. 2. Surface pressure ($mN.m^{-1}$) over time (s) between rapeseed oil and Ala − C (A) and Ala − B (B) proteins treated at different pH (—) 3.0, (—) 6.0, (—) 9.0 and temperature of heat treatment (♦) 25 °C, (■) 65 °C, (▲) 95 °C for 15 min. Data represent the mean of 3 measurements and standard deviation vary between 0.02 and 0.46.

3.2. Emulsifying properties

3.2.1. Emulsifying activity and stability indices

Fig. 3 represents the emulsifying activity and stability indices (EAI and ESI respectively) of Ala − C and Ala − B stabilised emulsions as a function of pH and heat treatment. EAI reflects the relative surface coverage of the protein on the oil droplet within a dilute emulsion and the droplet size. Conversely, ESI is an estimation of the relative stability of the emulsion after a predetermined time (Lam & Nickerson, 2015a).

Regarding EAI (Fig. 3A and B), both proteins display a different behaviour as a function of pH and treatment temperature, but they exhibit comparable maximum values, between 100 and 110 $m^2 g^{-1}$. EAI of Ala − B stabilised emulsions shifts limitedly between 84 and 110 $m^2 g^{-1}$ (Fig. 3B), while EAI of Ala − C stabilised emulsions (Fig. 3A) declines to 22 $m^2 g^{-1}$ at pH 6.0, where only the effect of pH is significant ($p < 0.001$).

Similar trends had already been observed for cow proteins (Lam & Nickerson, 2015a; Zhai et al., 2012): actually, it depends on the native state of the protein (apo or holo). For example, Ala − B in the holo − -form (calcium binding) was found to be more resistant to conformational changes in response to pH and temperature pre-treatments than the apo − form. As a result, higher droplet surface coverage (EAI) was obtained for the apo than the holo − form of Ala − B. Even though both proteins, Ala − C and Ala − B, are in their apo − form in this work (no calcium present or added in the commercial and prepared samples), structure flexibility is, therefore, widely different.

Besides, Voutsinas et al. (1983) reported that the change in emulsifying properties of whey proteins was reported to remain small against heat treatment, as in this work. However, Dissanayake and

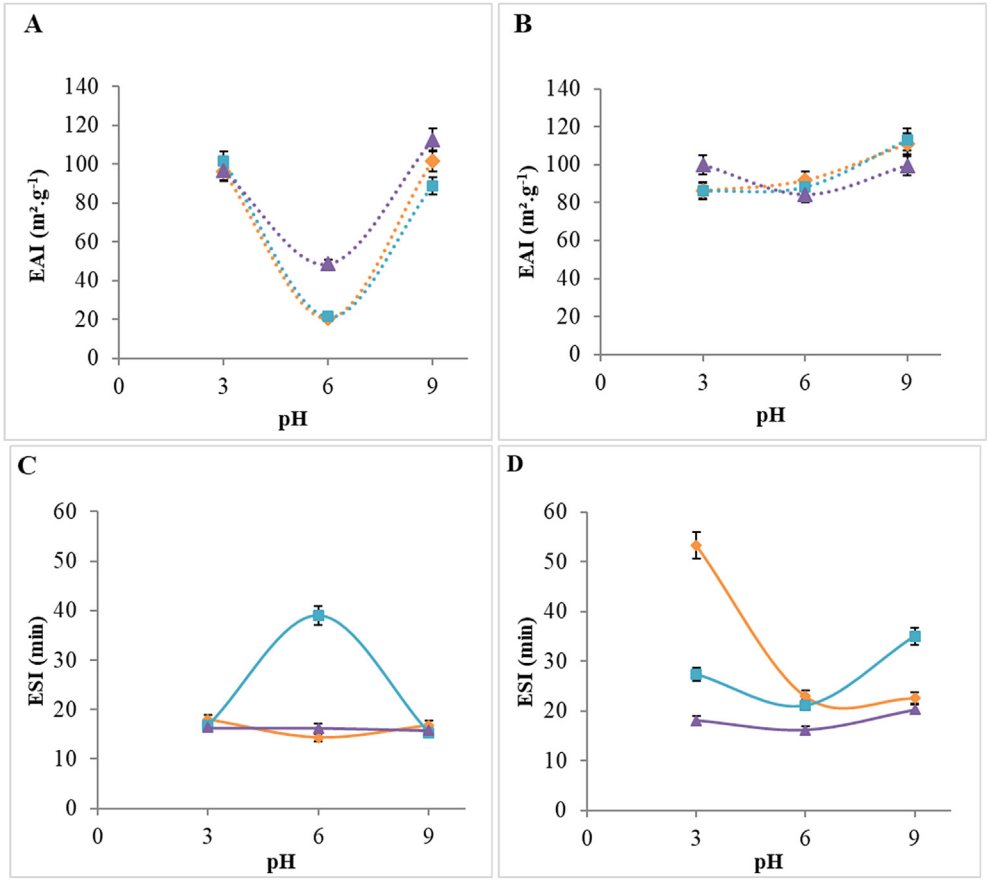

Fig. 3. Emulsifying Activity ($m^2.g^{-1}$) and Stability (min) Indices of Ala – C (A, C) and Ala – B (B, D) emulsions as a function of pH (3.0, 6.0 and 9.0) and temperature of heat treatment (♦) 25 °C, (■) 65 °C, (▲) 95 °C. Data represent the mean ± standard deviation (n = 3).

Vasiljevic (2009) stated, in contrast, that the EAI of bovine whey proteins increased with thermal treatment and denaturation due to chemical composition changes.

At pH 6.0, the adsorption of the Ala – C proteins at the oil interface seems to be ineffective, mainly due to the reduction of electrostatic repulsion between proteins close to their pI (5.1–5.2); thus, the proteins keep their globular conformation and cannot adhere to the oil – water interface under the dynamic conditions of emulsion preparation. In contrast, when EAI was greater (pH 3.0 and pH 9.0), Ala – C proteins are at their charged state and even exhibit conformational changes, especially at 95 °C, as previously observed by higher surface hydrophobicity in section 3.1.

As for emulsifying stability, Fig. 3D shows that ESI values of Ala – B stabilised emulsions are relatively higher than Ala – C (Fig. 3C), where maximum stability is estimated at 53 min and 35 min, respectively. The stability of protein – stabilised emulsions has been abundantly discussed in the literature (Dickinson, 2001; Lam & Nickerson, 2015b; Tcholakova, Denkov, Ivanov, & Campbell, 2006; Zhai et al., 2012) to be associated with electrostatic repulsion forces between droplets, steric stabilization by protein tails or loops on the oil droplet surface, and/or high viscosity of the continuous phase. It must be pointed out that opposite results have been reported on the effect of heat treatment on ESI for whey proteins (Dissanayake & Vasiljevic, 2009; Voutsinas et al., 1983). While Voutsinas et al. (1983) described an increase in ESI with heat treatment, Dissanayake and Vasiljevic (2009) advocated that no change in ESI was observed with increasing heat treatment temperature. However (Lam & Nickerson, 2015b), reported that the increased ability of the apo – form of Ala – B to adhere to the interface also allowed for greater emulsion stability. Stability was greater at pH 7.0 than pH 5.0 due to the presence of electrostatic repulsive forces between protein coated droplets. Furthermore, a pre-treatment at a temperature of 65 °C on Ala – B apo – form resulted in an increase in its secondary structure, leading to the greatest emulsion stability, presumably due to the partial denaturation and refolding at this temperature.

Finally, the stability of Ala – C stabilised emulsions at pH 3.0 and 9.0, exhibiting high EAI, can also be attributed to electrostatic forces. This conclusion is strengthened by the negligible effect of heat treatment on camel milk proteins as previously reported (Atri et al., 2010; Lajnaf et al., 2017b).

3.2.2. *Surface charge (ζ – potential)*

The stability of Ala – C and Ala – B stabilised emulsions after heat treatment and pH change was further assessed through ζ – potential measurements (Fig. 4). For both protein stabilised emulsions, the ζ – potential remains relatively constant regardless of the temperature. No significant effect of heat treatment ($p > 0.05$) was confirmed by ANOVA (Table 1). The ζ – potential of Ala – B stabilised emulsions (Fig. 4B) is relatively higher than Ala – C for which it remains between – 25 mV and – 40 mV and between – 15 mV and – 37 mV, respectively. Bhattacharjee (2016) reported that highly stable emulsion could be observed when ζ – potential departed from zero by about at least ± 30 mV. In this work, ζ – potential exceeds – 35 mV for both emulsions at specific values of pH and temperature (*i.e.* pH 9.0–25 °C/95 °C, and pH 6.0–65 °C/95 °C, pH 9.0–65 °C for Ala – C and Ala – B respectively), which highlights that electrostatic repulsions are involved in the emulsion stability by reference to ESI in section 3.2.1. The low net charge of emulsions at pH 3.0 is associated with positively charged Ala proteins below pI (5.2 and 4.2 for Ala – C and Ala – B, respectively). The significant effect of pH was higher for Ala – C than Ala – B stabilised emulsions according to ANOVA analysis (Table 1). Changes in surface charge lead the conformation structures of Ala – C to be more affected by pH change.

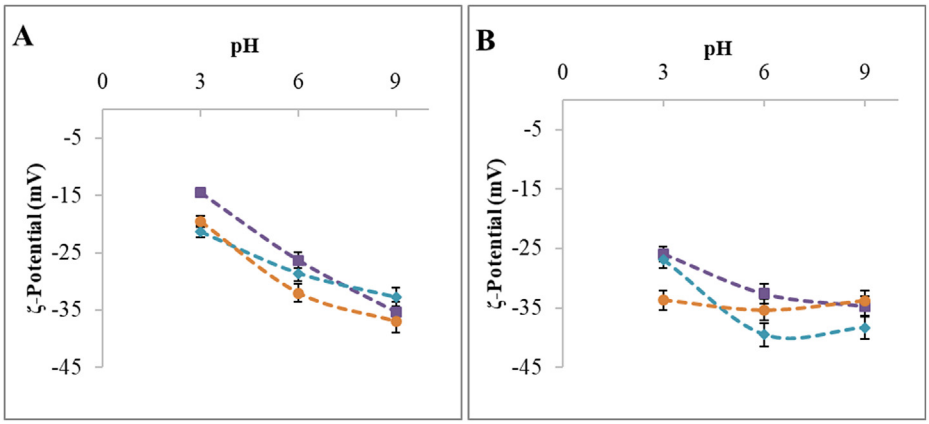

Fig. 4. ζ – Potential (mV) of Ala – C (A) and Ala – B (B) stabilised emulsions as a function of pH for different temperature of heat treatment (■) 25 °C, (♦) 65 °C, and (●) 95 °C. Data represent the mean ± standard deviation (n = 3).

Table 2
Sauter diameter d_{32} (μm) for Ala – C and Ala – B stabilised emulsions as a function of pH (3.0, 6.0, 9.0) and treatment temperature (25, 65, 95 °C). The letters "a", "b" and "c" represent homogenous subsets of different classes.

Temperature		25 °C	65 °C	95 °C
pH 3.0	Ala – C	3.95^{ba}	13.67^{b}	14.15^{bc}
	Ala – B	6.87^{ba}	4.89^{ba}	9.82^{b}
pH 6.0	Ala – C	2.00^{a}	1.43^{ab}	9.88^{ac}
	Ala – B	7.24^{ba}	6.53^{ba}	8.98^{b}
pH 9.0	Ala – C	8.93^{ba}	11.38^{b}	12.12^{bc}
	Ala – B	3.34^{a}	6.08^{a}	7.31^{ab}

3.2.3. Droplet size analysis

The variation of droplet size diameter (d_{32}) from image analysis and volume – based size distribution, as well as microscopic observations for Ala – C and Ala – B stabilised emulsion, as a function of the different pH and temperature treatment are presented in Table 2 and Fig. 5, respectively.

Overall, d_{32} for Ala – B and Ala – C stabilised emulsions, is ranging between 3.34 ± 0.01 μm and 9.8 ± 0.2 μm, and between 1.4 ± 0.04 μm and 14.2 ± 0.4 μm, respectively. It must, however, be pointed out that the smallest values of 1.4 and 2 μm, are observed for Ala – C stabilised emulsions when pH is 6.0, which corresponds to very low EAI values (Fig. 3A), *i.e.* large or flocculated droplets; thus, these emulsions had been subjected to creaming before high pressure homogenisation, and only a fraction of the oil phase was in the homogenized emulsions, which lead to a small number of very fine droplets. This result is supported by microscopic observations (Fig. 5) which highlight higher droplet density (i.e. number of droplets per picture) both at pH 3.0 and 9.0 with a similar diameter (Table 2), while a very low droplet density is observed at pH 6.0. Consequently, only the smallest droplets could be formed and stabilised at a pH close to pI. ANOVA indicates that the pH and the treatment temperature, as well as the interaction ($pH \times T$) for Ala – C proteins, are significant (Table 1). After heat treatment at 95 °C, all the d_{32} values of Ala – C stabilised emulsions reach their maximum values, which corresponds to high EAI (Fig. 3A). Larger droplets are formed and stabilised through hydrophobic interactions. Indeed, high surface hydrophobicity (Fig. 1A) was observed at this treatment temperature; equally, low interfacial tension was reported (Fig. 2A). It seems that, increasing the temperature of heat treatment at 95 °C usually increases significantly the Sauter diameter (d_{32}) of the droplets (Table 2) as in the case of the Ala – C stabilised emulsions, which also agrees with literature data on Ala – B reported after heat treatments at 80 °C and 90 °C (Zhai et al., 2012). However, for Ala – B stabilised emulsions, this increase in d_{32} at 95 °C of heat treatment could clearly be related to the decrease in ESI observed in Fig. 3D. Thus, protein denaturation induced by heat treatment shows a reduced capacity to form emulsified droplets close to pI when EAI remains low.

3.3. Emulsion rheology

The apparent viscosity (η) was measured vs. shear rate between 0.1 and 1000 s^{-1} and plotted in Fig. 6. The evolution of viscosity of Ala – C and Ala – B stabilised emulsions at a fixed shear rate (10 s^{-1}) is presented in terms of pH (3.0, 6.0 and 9.0) and temperature treatment (25 °C, 65 °C and 95 °C). First, all the studied emulsions exhibit a nearly Newtonian behaviour at a high shear rate, and non-Newtonian trends at low shear rate. The emulsion viscosity is lower for Ala – B (Fig. 6C) than Ala – C (Fig. 6A), and ranges between 2 mPa s and 40 mPa s, and between 1.5 mPa s and 87 mPa s, respectively. For Ala – B stabilised emulsions, the change in viscosity due to pH remains weak. Thus, the effect of the heat treatment is higher than the pH effect (Table 1). This relative invariability of the viscosity of Ala – B stabilised emulsions is correlated to EAI and ESI data presented in section 3.2. For Ala – C stabilised emulsions, only the effect of pH is significant ($p < 0.001$). The emulsion viscosity is higher at pH 3.0 than at pH 6.0 and 9.0, regardless of heat treatment. Some trends remain, however, similar for both proteins: pH 3.0 provided in general the highest values, and pH 9.0 the lowest ones. In terms of temperature, heat treatments at 95 °C and 65 °C lead to the lowest viscosity, while the highest is reported for unheated proteins (25 °C). For Ala – B stabilised emulsions, the effect of treatment temperature could be explained by changes in the conformational structure at the oil – water interface. At 25 °C, proteins adsorb in their native form and then unfold at the interface, mainly by hydrophobic interactions (Fig. 1B). A viscoelastic film is, therefore, formed around the oil droplets, resulting in high viscosity values. Conversely, after heat treatment at 65 °C and 95 °C of Ala – B proteins, their structure has already been modified by the temperature effect mainly through hydrophobic interaction, as shown previously, explaining the decrease in surface hydrophobicity at this temperature (Fig. 1B). Moreover, a slight shear-thickening region is observed between 20 and 100 s^{-1} (Fig. 6C).

As for Ala – C stabilised emulsions, the decrease in viscosity between pH 3.0 and 6.0 results from friction reduction caused by low droplet density, as shown by microscopic observations in Fig. 5A, as well as by the low surface coverage (EAI) as previously discussed (Fig. 3A). High viscosity at acidic pH is, therefore, consequent to attractive electrostatic forces between the two phases according to the low ζ – potential of camel protein emulsions previously reported (Fig. 4A) as well as to a reduced interfacial tension (Fig. 2A). Besides, an intermediate plateau emerged between 20 and 100 s^{-1}, included between the shear-thinning behaviour observed at low and vert high shear, i.e. before the high-shear Newtonian plateau; this can be due to the elastic behaviour of protein layers when compressed due to shear (Amin, Barnett, Pathak, Roberts, & Sarangapani, 2014). Several authors (Alvarez-Sabatel, Martínez de Marañón, & Arboleya, 2018; Dickinson, 2001; Liang, Patel, Matia-Merino, Ye, & Golding, 2013) had already reported that elastic interfacial layers, which is the case of adsorbed protein layers, could lead to shear-thickening trends. It was reported

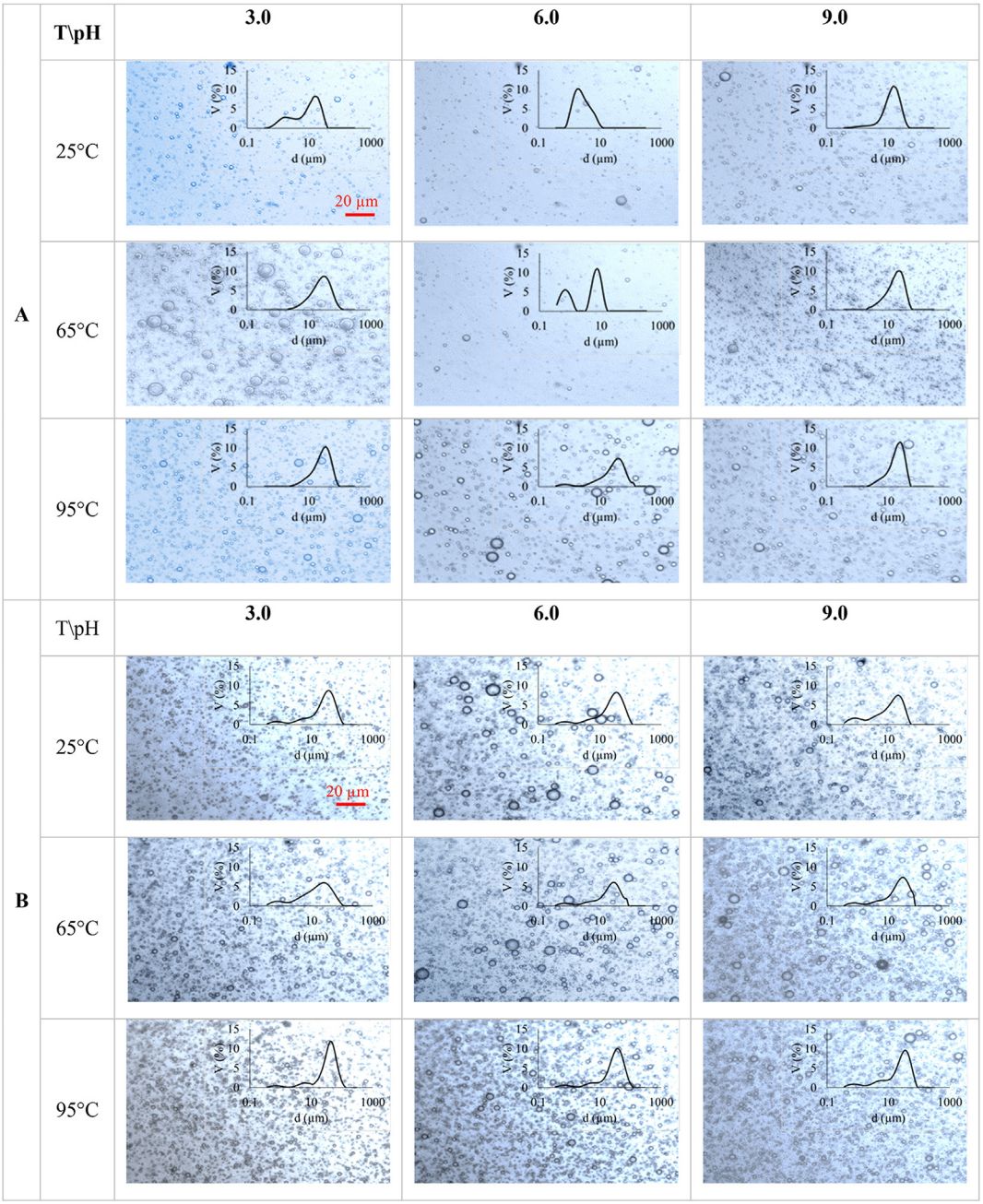

Fig. 5. Volume-size distribution (%) of oil droplet diameter (µm) and micrographs of emulsions stabilised by Ala − C (A) and Ala − B (B) proteins treated at different pH (3.0, 6.0, 9.0) and temperature (25, 65 and 95 °C for 15 min).

that interfacial tension plays also an important role in controlling the viscosity of dilute emulsions where high shear rate causes internal circulation inside the drops and drop deformation, and the interfacial tension minimizes the movement of the flow pattern outside the drops (Otsubo & Prud'homme, 1994).

4. Conclusions

Two different types of α − lactalbumin proteins from camel and cow milks have been compared as a function of pH change and heat treatment conditions; their behaviours at the oil − water interface have been deduced through direct and indirect analysis of their structures and their emulsifying properties. It has been identified that the major factor governing the camel α − lactalbumin emulsion stability is its flexibility enabling conformational rearrangement, which is affected by protein heat treatment, while electrostatic repulsion is still a key factor to explain the effect of pH change. Camel α − lactalbumin is significantly affected by pH variation while cow α − lactalbumin is more sensitive to heat treatment. High heat treatment enhances the camel α − lactalbumin surface properties, which maintains the emulsifying properties of this protein. Ala − C proteins decrease the interfacial tension by exposing its hydrophobic groups, especially at pH 3.0 after heat treatment at 95 °C. Emulsion activity and stability are affected by electrostatic repulsion for both proteins, especially for Ala − C. Thus, it is more difficult to prepare and stabilise emulsions close to the pI, *i.e.* when pH is about 6.0, with Ala − C than with Ala − B, due to reduced hydrophobic interaction. Compared to cow α − lactalbumin, the major factor that differs from camel α − lactalbumin is, therefore, steric hindrance which behaves differently as a function of environmental changes. These facts suggest an unbalance in the two dominant forces

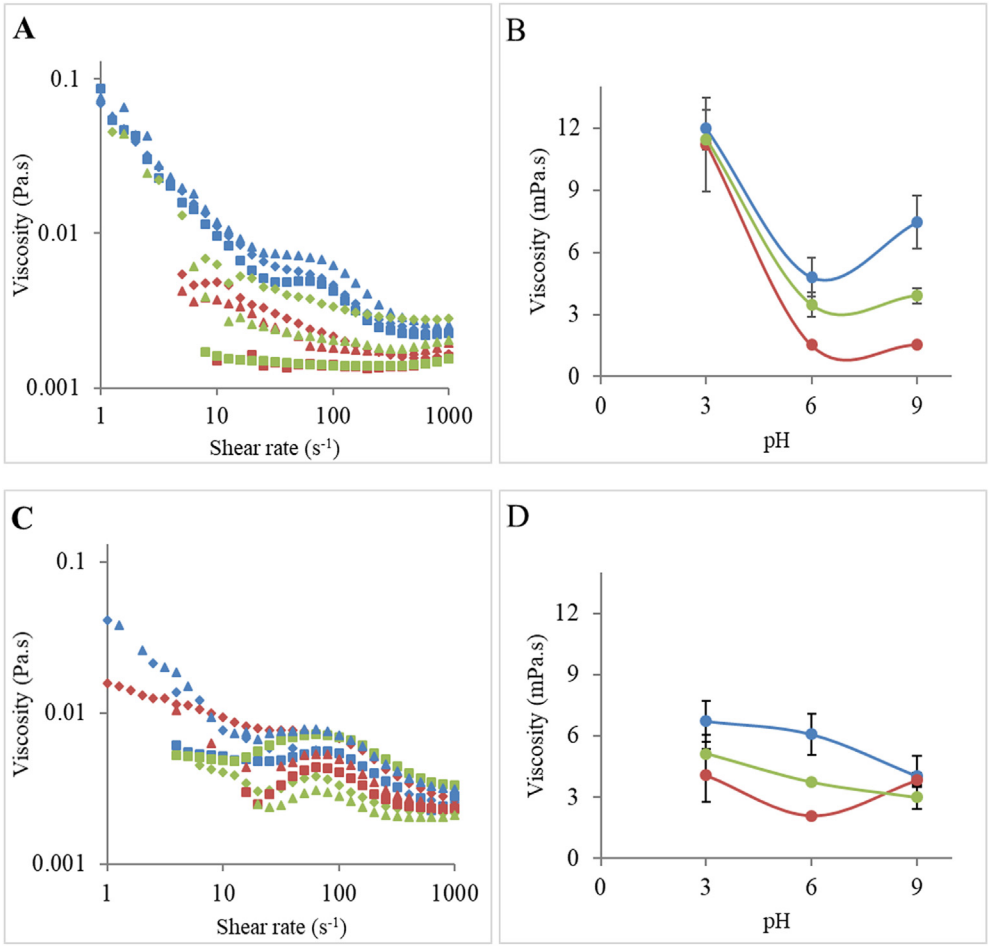

Fig. 6. Apparent viscosity (Pa.s) vs. shear rate (A, C) respectively for Ala – C and Ala – B stabilised emulsions in terms of pH (●) 3.0, (●) 6.0, (●) 9.0 and temperature of heat treatment (♦) 25 °C, (■) 65 °C and (▲) 95 °C and at a fixed shear rate (B, D) respectively for Ala – C and Ala – B stabilised emulsions in terms of pH for different treatment temperature (——) 25 °C, (——) 65 °C, (——) 95 °C. Data represent the mean ± standard deviation (n = 3).

(the electrostatic effect and configurational entropy) that drives conformational rearrangement at emulsion interfaces toward the electrostatic repulsion. These results thus broaden our understanding of the factors controlling protein structural change at emulsion interfaces and how this affects emulsion stability. Depending on the desired application (high/low stability, high/low emulsifying activity, high/low viscosity …), it is possible to adjust the main factors (i.e. pH, temperature and protein type) to obtain desired functionality.

Conflict of interest and authorship conformation form

- ✓ All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.
- ✓ This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.
- ✓ The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript
- ✓ The following authors have affiliations with organizations with direct or indirect financial interest in the subject matter discussed in the manuscript:

Acknowledgements

This project was financially supported by the higher education and scientific research ministry of Tunisia. Experiments were conducted at Institut Pascal (UMR CNRS-UCA-Sigma Clermont 6602), GePEB group in Clermont-Fd (France). The author wants to thank Dr. Roua Lajnaf and Dr. Ahmed Zouari for proofreading.

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