



The effect of pH and temperature pre-treatments on the structure, surface characteristics and emulsifying properties of alpha-lactalbumin



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ABSTRACT

The effect of pH (5.0 or 7.0) and temperature (25.0, 65.0 and 95.0 °C) on the physicochemical and emulsifying properties of type-1 (with calcium, ALA-1) and type-3 (without calcium, ALA-3) alpha-lactalbumin (ALA) were examined. By increasing the temperature of pre-treatment, changes in ALA conformation allowed for greater surface hydrophobicity and caused changes in its surface charge. pH also influenced surface charge for ALA where enhanced repulsion at pH 7.00 was observed resulting in reduced aggregation despite having greater hydrophobicity. Findings indicate that changes to protein conformation using various pH and temperature pre-treatments influenced their surface chemistry, aggregation and ability to align at the oil–water interface. Overall, emulsions were found to be more stable at pH 7.0 than 5.0 due to the greater amount of electrostatic repulsive forces between droplets present at pH 7.0. Under the conditions examined in this study, ALA-3 pre-treated at 65 °C and at pH 7.00 resulted in the best emulsifying properties.

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1. Introduction

Alpha-lactalbumin (ALA) is the second major protein found in bovine whey next to beta-lactoglobulin. Its biological function in milk is to aid with lactose biosynthesis however, it is also highly nutritious and functional as a food ingredient (Berliner, Meinholtz, Hirai, Musci, & Thompson, 1991; Bonnaillie & Tornasula, 2012; Heine, Klein, & Reeds, 1991; Korhonen & Pihlanto, 2007). The functionality of ALA has been studied in terms of its foaming, gelling and emulsifying properties (Hunt & Dalgleish, 1994; Ibanoglu & Ibanoglu, 1999; Matsudomi, Oshita, Kobayashi, & Kinsella, 1993; Zhai et al., 2012). The latter represents the primary focus of this study. Emulsions are formed when two immiscible liquids are mechanically agitated whereby one becomes dispersed as droplets within the other. Proteins, which are amphiphilic in nature, serve to help stabilize the emulsion by decreasing the surface tension between the two phases. ALA also has been reported to change its secondary and tertiary structure upon adhering to the oil–water interface to enhance emulsion stability (Zhai et al., 2012). Hunt and Dalgleish (1994) reported that at pH < 7.0, ALA preferentially absorbs to the oil–water interface over beta-lactoglobulin (β-LG).

ALA is a globular protein that has a molecular mass of 14–16 kDa and an isoelectric point of 4.2. Structurally it is comprised of two domains: a α -domain made of 4 α -helices and a β -domain made of β -sheets and looped regions (Bramaud, Aimar, & Daufin, 1995; Wang & Lucey, 2003; Wu, Shulman, Peng, & Kim, 1996). In general, the presence of bound calcium leads to a less flexible protein with enhanced thermal stability (Hendrix, Griko, & Privalov, 2000; Rajaraman, Raman, Ramakrishna, & Mohan Rao, 1998; Relkin, Launay, & Eynard, 1993). For instance, ALA's denaturation temperature was reduced from ~64 °C to ~35 °C once calcium was removed (Relkin et al., 1993). Hendrix et al. (2000) reported that increasing the CaCl₂ content from 0.0 to 2.0 mM CaCl₂ caused an increase in the enthalpy of unfolding from 271 to 318 kJ mol^{−1}. Solvent conditions, such as pH and temperature have also been found to alter the conformation of ALA (Cornec, Kim, & Narsimhan, 2001). For instance at extreme pH (e.g., 2.0), ALA has been found to change in conformation to a more open structure (Cornec et al., 2001). Fang and Dalgleish (1998) reported that when ALA is exposed to temperatures >90 °C, irreversible denaturation occurs, whereas at temperatures <90 °C, refolding of the protein conformation can occur.

The overall goal of this study is to investigate the effect of pH and temperature pre-treatments on the emulsifying properties of type-1 (with calcium) and type-3 (without calcium) ALA. Understanding the emulsifying properties of ALA may help scientists and industry design novel functional foods (i.e. infant formulas).

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using ALA as an encapsulating agent for the delivery of bioactives and or other nutraceuticals.

2. Materials and methods

2.1. Materials

Type-1 (with calcium) and type-3 (without calcium) ALA were purchased from Sigma–Aldrich (Oakville, ON, Canada) and used without further purification. Henceforth, the two types of ALA will be referred to as ALA-1 and ALA-3. A certificate of analysis was provided by Sigma–Aldrich indicating the calcium content for ALA-1 (Product #: L5385, Lot #: 110M7003V) and ALA-3 (Product #: L6010, Lot #: 110M7029V) to be 2 and 0.3 mol Ca^{2+} per mole of ALA, respectively. Protein content for ALA-1 and ALA-3 were determined to be 87.28% and 83.23% respectively ($\%N \times 6.38$) as measured using a micro-Kjeldahl digester (Micro Digestor, Labconco, Kansas City, MO, USA) and distillation unit (RapidStill I, Labconco, Kansas City, MO, USA) according to AOAC Official Method 920.87 (AOAC, 2003). Canola oil used in this study was purchased from a local supermarket. All other chemicals used in this study were of reagent grade and purchased through Sigma–Aldrich (Oakville, ON, Canada). The water used in this research was filtered using a Millipore Milli-Q™ water purification system (Millipore Corp., Milford, MA, USA).

2.2. Sample preparation

ALA stock solutions (0.1%, w/w corrected for protein content) were prepared by dispersing the ALA-1 and ALA-3 powder in water adjusted to pH 5.0 or 7.0 using either 0.5 M HCl or 0.5 M NaOH, followed by mechanically stirring (500 rpm) at room temperature (21–23 °C) for 60 min. Protein solutions were then poured into screw capped 15 mL centrifuge tubes to prevent moisture losses, and held in a water bath for 30 min at 25 °C, 65 °C and 95 °C. Tubes were left out to cool down to room temperature prior to analysis.

2.3. Circular dichroism

Circular dichroism (CD) was performed on 0.02% (w/w) ALA-1 and ALA-3 solutions using a PiStar-180 spectrometer (Applied Photophysics, Surrey, UK) equipped with a 75 W Mercury Xenon Lamp. CD was performed on samples contained in quartz cuvettes with a 0.5 mm path length. A CD spectrum was taken from 190 to 240 nm at intervals of 0.5 nm where the slit width was set at 6.0 nm. A spectrum for the mean residue molar ellipticity ($\text{deg cm}^2 \text{dmol}^{-1}$) was plotted as a function of wavelength for all samples. A comparison in spectral data was made to show relative changes to the secondary structure among sample treatments whereby the flattening of the CD spectra indicates loss in structure.

2.4. Surface hydrophobicity

The surface hydrophobicity for each protein solution was determined by intrinsic fluorescence as a function of emission wavelength. A FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, N.J., USA) was used to determine the intrinsic fluorescence for 0.05% (w/w) protein solutions at a constant excitation wavelength of 275 nm as a function of emission wavelength between 285 to 450 nm using a 1 nm slit width. All intensity data was arbitrarily divided 10,000 prior to statistical analysis and graphing. This technique measures the fluorescence of the aromatic amino acids tyrosine, tryptophan and phenylalanine. The maximum fluorescence intensity (in arbitrary units (a.u.)) was determined for each spectra captured.

2.5. Surface charge

The electrophoretic mobility (U_E) was determined using the Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA) for all protein samples (0.05%, w/w). The zeta-potential (ζ ; units: mV) was determined from the electrophoretic mobility (U_E) using Henry's equation (Eq. (1)):

$$U_E = \frac{2\varepsilon f(\kappa\alpha)}{3\eta} \quad (1)$$

where ε is the permittivity (units: F (Farad)/m), $f(\kappa\alpha)$ is the function related to the ratio of particle radius (α ; units: nm) and Debye length (κ ; units: nm^{-1}), and η is the dispersion viscosity (units: mPa s) (McClements, 2005). The Smoluchowski approximation $f(\kappa\alpha)$ was set as 1.5, as is accustom for folded capillary cells and, with particles larger than 0.2 μm dispersed in moderate electrolyte concentration ($>1 \text{ mM}$). The Smoluchowski approximation assumes that (a) concentration of particles (proteins) is sufficiently high such that such thickness of the electric double layer (Debye length) is small relative to the particle size ($\kappa\alpha \gg 1$); and (b) ζ is linearly related to U_E .

2.6. Hydrodynamic radius

The hydrodynamic radius for ALA aggregates formed under the different pH and temperature pre-treatment conditions were measured using the Zetasizer Nano-ZS90 (Malvern Instruments, Westborough, MA, USA). Approximately, 1 mL of each protein solution (0.05%, w/w) was placed in a plastic cuvette (1 cm path length) within the Zetasizer's sample holder. The scattering intensity was measured at a 90° angle at 633 nm using a 4 mW He/Ne laser at room temperature (21–23 °C). The hydrodynamic radius was estimated based on the Stokes–Einstein equation (Eqs. (2) and (3)):

$$D = \frac{k_B T}{6\pi\eta r} \quad (2)$$

which may be re-written as:

$$r = \frac{k_B T}{6\pi\eta D} \quad (3)$$

where, k_B is the Boltzmann constant ($1.38062 \times 10^{-23} \text{ J K}^{-1}$), D is the self-diffusion constant ($\text{m}^2 \text{s}^{-1}$), T is the absolute temperature (K), η is viscosity of the medium ($\text{kg m}^{-1} \text{s}^{-1}$) and r is the radius of the diffusing particle (m) (Hoffman et al., 1998).

2.7. Interfacial tension

The interfacial tension for each protein solution (0.1%, w/w) was measured using a Lauda TD 2 Tensiometer (Lauda-Königshofen, Germany) equipped with a Du Nüoy ring (20 mm diameter). Within a 40 mm diameter glass sample cup, a 20 mL protein solution was added, followed by the immersion of the Du Nüoy ring and then the addition of upper canola oil layer (20 mL). The ring was then pulled upwards to stretch the interface to measure the maximum force without breaking into the oil phase. Samples were measured at 3 min intervals until three consecutive measurements obtained a standard deviation of less than 0.1 mN/m. Interfacial tension was calculated from the maximum force (F_{max} ; units: milli-Newtons; instrument measures $\text{mg} \times \text{gravity}$) using the following equation (Eq. (4)):

$$\gamma = \frac{F_{\text{max}}}{4\pi R\beta} \quad (4)$$

where, γ is the interfacial tension (mN/m), R is the radius of the ring (20 mm), β is a correction factor that depends on the dimensions of the ring and the density of the liquid involved (McClements, 2005).

2.8. Emulsification stability and activity indices

Emulsions were prepared by mixing 5 g of the 0.1% (w/w) ALA solution with 2 g of canola oil within a 50 mL plastic centrifuge tube, followed by homogenisation at 7200 rpm for 5 min using an Omni Macro Homogenizer (Omni International Inc., Marietta, GA) equipped with a 20 mm saw tooth generating probe. Emulsion stability (ESI) and activity (EAI) indices were determined according to Pearce and Kinsella (1978). The EAI provides an estimate of the relative surface coverage of a protein on an oil droplet within a dilute emulsion, whereas the ESI gives an estimate of its relative stability after a pre-determined time. Immediately after emulsion formation, a 50 μ L aliquot is transferred into 7.5 mL of pH-adjusted water containing 0.1% sodium dodecyl sulphate (SDS), vortexed for 10 s, and then absorbance read at 500 nm using a ultraviolet–visible spectrophotometer (Genesys 20, Thermo Scientific, Madison, WI) and plastic cuvettes (1 cm path length). A second aliquot of the emulsion was then taken after 10 min, following the same procedure. The EAI and ESI were determined using Eqs. (5) and (6), respectively:

$$\text{EAI}(\text{m}^2/\text{g}) = \frac{2 \cdot 2.303 \cdot A_0 \cdot N}{c \cdot \varphi \cdot 10000} \quad (5)$$

$$\text{ESI}(\text{min}) = \frac{A_0}{\Delta A} \cdot t \quad (6)$$

where A_0 is the absorbance of the diluted emulsion immediately after homogenisation, N is the dilution factor (150 $\times$), c is the weight of protein per volume (g/mL), φ is the oil volume fraction of the emulsion, ΔA is the change in absorbance between 0 and 10 min ($A_0 - A_{10}$) and t is the time interval, 10 min.

2.9. Statistics

All experiments were performed in triplicate and reported as the mean $\pm$ one standard deviation. A three-way analysis of variance (ANOVA) was performed to test for significance in the main effects of type (ALA-1 and ALA-3), pH (5.0, 7.0) and heat pre-treatment conditions (25, 65, 95 $^{\circ}$ C), along with their associated interactions on the physicochemical and emulsifying properties of ALA. Statistical analyses were performed with Systat (SPSS Inc., Ver. 10, 2000, Chicago, IL).

3. Results and discussion

3.1. Protein conformation

Circular dichroism was used to measure relative changes in ALA's secondary structure in response to pH and temperature pre-treatments for both ALA-1 and ALA-3 (Fig. 1). Overall, mean molar ellipticity data for ALA-1 was more stable against temperature pre-treatments than ALA-3 presumed due to the presence of calcium. At pH 5.00, there was very little change in molar ellipticity as a function of temperature for both types of ALA (Fig. 1A). However at pH 7.00, ALA-1 underwent increased ordering in the secondary structure as temperatures increased from 25 to 65 $^{\circ}$ C as evident by a decreased mean ellipticity curve (Fig. 1B). As temperatures increased to 95 $^{\circ}$ C, a loss in secondary structure was then observed as evident by a rise in the mean ellipticity curve (higher than both the 25 and 65 $^{\circ}$ C). It is hypothesised that at 65 $^{\circ}$ C, the protein experiences increased conformational entropy allowing for the polypeptide chains to undergo greater ordering. However at 95 $^{\circ}$ C, protein denaturation is thought to occur as the result of the breakage of hydrogen bonds at higher temperatures leading to a loss in structure. Similarly, it has been previously reported that

temperatures above 90 $^{\circ}$ C, ALA becomes irreversibly denatured (Fang & Dalgleish, 1998).

Conformational re-ordering have been previously reported in soy proteins (Miriani, Keerati-u-rai, Corredig, Iametti, & Bonomi, 2011), rice proteins (Ellepola & Ma, 2005) and ALA (Griko, 1999). Ellepola and Ma (2005) reported that rice proteins underwent partial denaturation depending on the time proteins were subjected to thermal treatment. They reported that by increasing the thermal pre-treatment on rice proteins, an increase in the denaturation temperature was observed which was complemented by a decrease in its denaturation enthalpy (Ellepola & Ma, 2005). Similarly, Griko (1999) reported a similar increase in denaturation temperature in ALA samples pre-treated at various temperatures. Furthermore, Griko (1999) stated that these changes in denaturation temperature were largely dependent on solvent conditions. Using various spectroscopic techniques, Miriani et al. (2011) reported tertiary structural re-orientation of soy proteins due to various temperatures of thermal treatment.

Biologically, calcium is known to bind to ALA to aid in the biosynthesis of lactose (Berliner et al., 1991). The effect of calcium ions on the thermal properties of ALA has been well studied (Atri et al., 2010; Relkin et al., 1993; Wehbi et al., 2005). It has been reported that the presence of calcium does not greatly affect the structure of ALA as it relates to the retention of the shape of the ellipticity curve as seen in Fig. 1A and B, and as reported by others (Kuwajima, Harushima, & Sugai, 1986; Pfeil & Sadowski, 1985). Cornec et al. (2001) reported a similar ellipticity curve for native ALA at pH 7.5 in a 20 mM phosphate buffer at 25 $^{\circ}$ C. Calcium has been found to enhance the thermal stability of ALA when its concentration increased in solution (Hendrix et al., 2000; Relkin et al., 1993). Hendrix et al. (2000) reported that the enthalpy associated with denaturation increased with calcium concentration which depended on the degree of saturation of calcium ions on ALA.

In the case of ALA-3, the mean molecular ellipticity curves appeared to be similar at both pH 5.00 and 7.00. ALA-3 greatly increased structuring by a decreased in mean ellipticity as temperatures were raised from 25 to 65 $^{\circ}$ C which was followed by an increase in mean ellipticity as temperatures reached 95 $^{\circ}$ C indicating a loss of structure (Fig. 1C and D). The larger extent of conformational changes in ALA-3 compared to ALA-1 is attributed to ALA-3 structure under these solvent and thermal conditions. It is speculated that the absence of calcium ions in ALA-3 can undergo greater changes to its structure in response to temperature pre-treatments, and possibly leading to a partially unravelled state depending on the solvent conditions. A partially unravelled state could also show increased surface hydrophobicity (Arai & Kuwajima, 1996), form aggregates (Rajaraman et al., 1998) and have pronounced hydrogen bonding (Arai & Kuwajima, 1996) relative to the native state. The pronounced hydrogen bonding might explain for the enhanced ordering seen with ALA-3 treated at 65 $^{\circ}$ C. As ALA returns to room temperature, it re-folds from its unravelled state (Kuwajima, 1996; Permyakov & Berliner, 2000) sometimes into a non-native state (Hong & Creamer, 2002; Rajaraman et al., 1998). From Fig. 1, ALA-3 at 65 $^{\circ}$ C suggests that its structure is not in its native conformation.

3.2. Surface characteristics

The surface charge of ALA-1 and ALA-3 as a function of temperature pre-treatments and solution pH was determined by measuring the protein's zeta potential (Fig. 2). A 3-way ANOVA determined that the main factors of protein-type ($p < 0.001$) and pH ($p < 0.001$) as well and the interactions pH $\times$ temperature ($p < 0.001$), pH $\times$ protein-type ($p < 0.05$) and temperature $\times$ type ($p < 0.05$) were statistically significant (Table 1). At pH 5.00, both

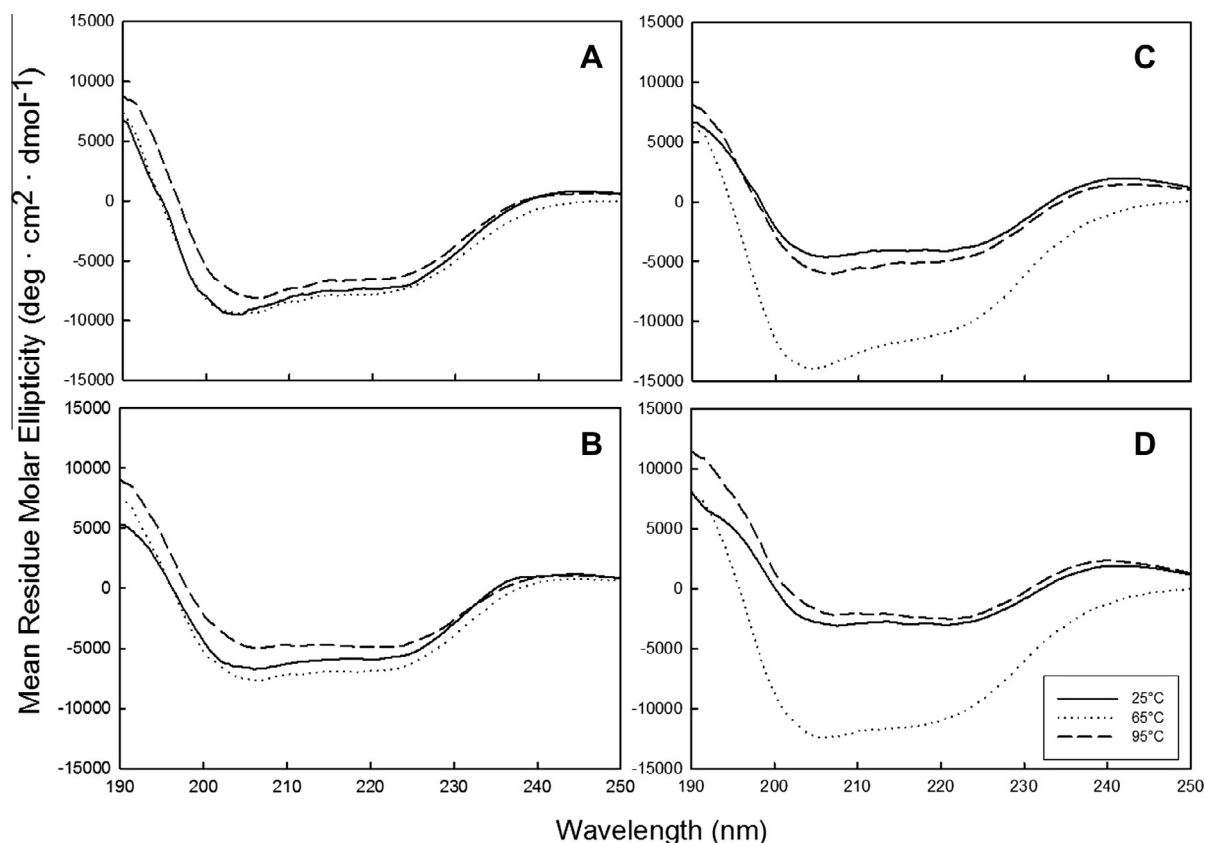


Fig. 1. Mean residue molar ellipticity data for ALA-1 (A, B) and ALA-3 (C, D) at pH 5.00 (A, C) and 7.00 (B, D) as a function of wavelength and temperature pre-treatments. Curves represent the means of 4 spectrums.

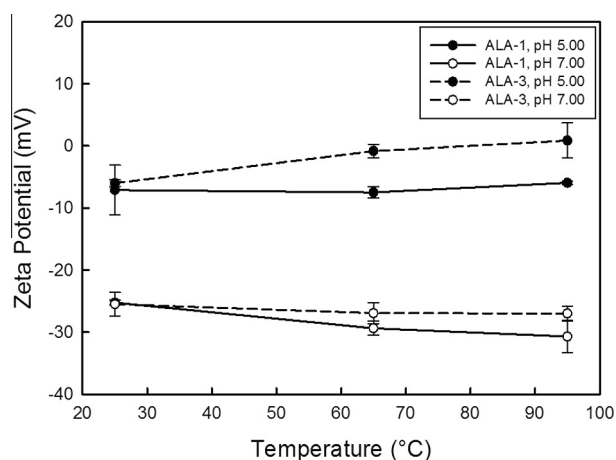


Fig. 2. Zeta potential of ALA-1 and ALA-3 proteins as a function of pH and temperature pre-treatments. Data represent the mean $\pm$ one standard deviation ($n = 3$).

ALA-1 and ALA-3 carried a low net charge regardless of the temperature. In the case of ALA-1, the zeta potential remained relatively constant regardless of the temperature pre-treatment at ~ -6.83 mV, whereas for ALA-3 the zeta potential rose slightly from ~ -5.99 mV at 25 °C to $\sim +0.87$ mV at 95 °C (Fig. 2). The low net surface charge at pH 5.00 is believed to be associated with the proximity to ALA's isoelectric point (pI 4.2 (Bramaud et al., 1995; Wang & Lucey, 2003; Wu et al., 1996)). At pH 7.00, ALA-3 remained constant regardless of the temperature at ~ -26.4 mV, whereas ALA-1 was much more temperature dependent. For

instance, at 25 °C the zeta potential was ~ -25.2 mV, then declined significantly to ~ -29.4 mV at 65 °C where it remained relatively constant to 95 °C (~ -30.7 mV) (Fig. 2). Zeta potential values were highly negative at pH 7.00 since it is away from its pI value (pH 4.2). The factors which affected the surface charge were dominated by the pH effects on these proteins. Overall, it was observed that in the absence of calcium, changes in the conformation of ALA-1 caused the surface charge to be more negative than ALA-3 when these proteins were treated at higher temperatures.

The surface hydrophobicity of ALA-1 and ALA-3 were determined by measuring the intrinsic fluorescence of exposed hydrophobic amino acid residues tryptophan, phenylalanine and tyrosine in the solvent. The maximum fluorescent intensity was used for comparative purposes as a function of protein-type, temperature and pH (Fig. 3). A 3-way ANOVA indicated that the three main factors (protein-type, pH and temperature) and all its subsequent interactions were significantly different ($p < 0.001$) (Table 1). Overall, fluorescent intensity was greater for ALA-3 than ALA-1, and at pH 7.00 than pH 5.00 (Fig. 3) indicating that at pH 7.00 proteins has a more open structure with exposed hydrophobic moieties than at a pH closer to its pI value. And that ALA-3 was structurally more open than ALA-1 presumed due to the lack of calcium.

ALA-3 at pH 7.00 was found to show the greatest fluorescent intensity (~ 66.5 a.u.) and was found to be temperature independent (Fig. 3). These findings complement zeta potential data which also was found to be temperature independent (Fig. 2). ALA-3 at pH 5.00, displayed the next highest hydrophobicity, however was more temperature dependent (Fig. 3). For instance at 25 °C maximum intensity was found at ~ 52.1 a.u., which then decreased to ~ 48.9 a.u. with a 65 °C temperature pre-treatment, before

Table 1

Statistical results from a 3-way analysis of variance describing the physicochemical and emulsifying properties ALA proteins as a function of pH (5.00 vs. 7.00) (A), temperature pre-treatments (25 °C vs. 65 °C vs. 95 °C) (B) and protein type (ALA-1 vs. ALA-3) (C). Note, NS means 'not significant' referring to a *p*-value > 0.05.

Property	Main Factors			Interactions			
	pH (A)	Temp (B)	Type (C)	A × B	A × C	B × C	A × B × C
<i>(a) Physicochemical properties</i>							
Hydrophobicity	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001
Zeta potential	<i>p</i> < 0.001	NS	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.05	<i>p</i> < 0.05	NS
Hydrodynamic radius	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001
Interfacial tension	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	NS	NS	<i>p</i> < 0.001	NS
<i>(b) Emulsifying properties</i>							
Emulsification activity index	<i>p</i> < 0.001	NS	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	NS	NS
Emulsification stability index	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001	<i>p</i> < 0.001

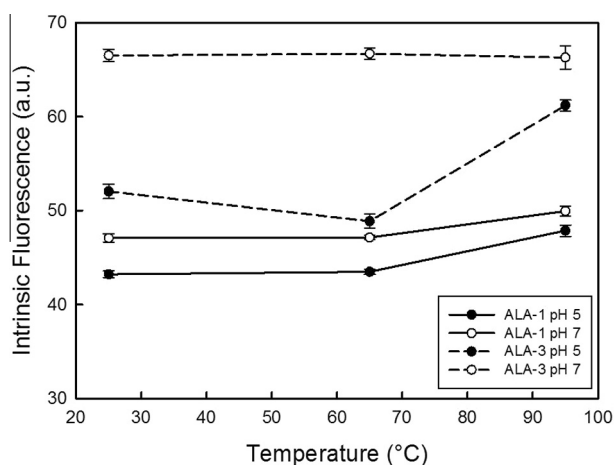


Fig. 3. Maximum fluorescent intensity of ALA-1 and ALA-3 proteins as a function of pH and temperature pre-treatments. Data represent the mean $\pm$ one standard deviation ($n = 3$).

increasing to ~ 61.2 a.u. at 95 °C which suggests a change in the tertiary structure of the protein (Fig. 3).

In the case of ALA-1, the temperature effect was similar where maximum fluorescent intensity was constant when temperatures were at 25 °C and 65 °C occurring at ~ 43.4 a.u. and ~ 47.1 for solvent pH of 5.00 and 7.00, respectively (Fig. 3). Fluorescent intensity then increased to ~ 47.9 a.u. and ~ 50.0 a.u. at solvent pH of 5.00 and 7.00, respectively as temperatures were raised to 95 °C (Fig. 3). The rise in fluorescence reflects a change in tertiary structure conformation due to thermal denaturation where an increased amount of hydrophobic moieties become exposed attributed to the denaturation of ALA-1 at temperatures above 90 °C (Fang & Dalglish, 1998).

The presence of calcium was previously been reported to not affect the secondary structure of ALA (Kuwajima et al., 1986; Pfeil & Sadowski, 1985). Therefore, in the present study it is hypothesised that observed changes in hydrophobicity may be attributed to the effect of calcium on the tertiary structure of ALA. In the absence of calcium, ALA-3 obtained an open conformation which allowed for greater exposure of the hydrophobic moieties normally shielded away in the presence of calcium (i.e. in ALA-1). This explains for the greater surface hydrophobicity in ALA-3 at all temperatures and pH conditions compared to ALA-1.

3.3. Protein aggregation

The hydrodynamic radius for ALA aggregates as a function of protein-type, pH and temperature pre-treatments were investigated by dynamic light scattering. A 3-way ANOVA determined

that for all these main factors and subsequent interactions were found to be statistically significant ($p < 0.001$) (Table 1). ALA-3 at pH 5.00 showed the greatest size and temperature dependence relative to the other samples indicating an increased amount of protein–protein interactions which was attributed to very weak net charge over the complete temperature range (~ -5.99 mV at 25 °C to ~ 0.874 mV at 95 °C) (Fig. 2) and relatively high hydrophobicity relative to the ALA-1 samples (at pH 5.00 and pH 7.00) (Fig. 3). The lack of calcium to stabilize the ALA structure and the lack of surface charge is thought to lead to a more open structure than the other samples, which promotes protein–protein aggregation. The hydrodynamic radius was found to increase from ~ 503.3 nm at pH 25 °C to ~ 1064 nm at 65 °C where it remained constant still at ~ 948.7 nm at 95 °C (Fig. 4). Similar behaviour was seen for ALA-3 at pH 7.00, and for ALA-1 at both pH 5.00 and 7.00, except the overall magnitude of the hydrodynamic radius was ~ 200 nm or less. In all 3 samples, the radius increased slightly from 25 to 65 °C where it then remained constant even at 95 °C (Fig. 4).

Various authors have studied the denaturation and aggregation behaviour of ALA (Bertrand-Harb, Baday, Dalgallarrondo, Chobert, & Haertle, 2002; Chaplin & Lyster, 1986; McGuffey, Epting, Kelly, & Foegeding, 2005). The ability for ALA to form aggregates depends on the protein concentration, heating profile and solvent properties (Bertrand-Harb et al., 2002; Chaplin & Lyster, 1986; McGuffey et al., 2005). ALA was found to denature when exposed to heat and refolded upon cooling (Kuwajima, 1996; Permyakov & Berliner, 2000). The degree of refolding depends on the aforementioned variables where one group reported that at temperatures above 90 °C, irreversible denaturation was observed

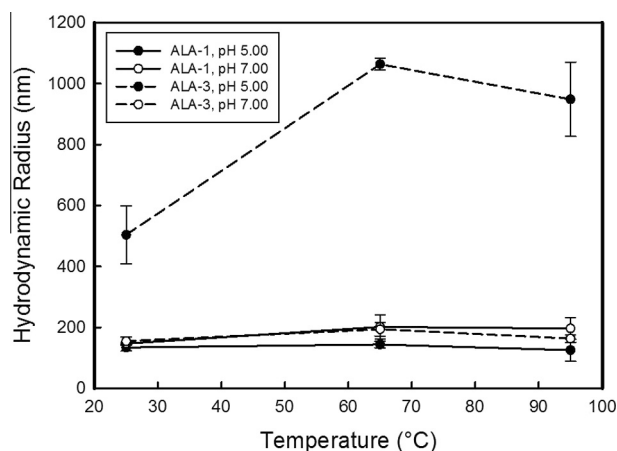


Fig. 4. Mean hydrodynamic radius (nm) of ALA-1 and ALA-3 proteins as a function of pH and temperature pre-treatments. Data represent the mean $\pm$ one standard deviation ($n = 3$).

(Chaplin & Lyster, 1986; McGuffey et al., 2005). The heating of ALA at 95 °C was reported to induce the exposure of thiol groups which caused ALA to aggregate (McGuffey et al., 2005). When thiol groups were reduced, hydrophobic interactions caused large aggregates to form (McGuffey et al., 2005). This explains for the large aggregates observed in Fig. 4. The larger aggregates observed at 65 and 95 °C compared to those at 25 °C may be attributed to the exposure of thiol groups leading to protein–protein interactions and aggregate formation. The size of these aggregates are limited and speculated to be affected by the strong electrostatic repulsion experienced by ALA at pH 7.00. Similarly, Tcholakova, Denkov, Ivanov, and Campbell (2006) have reported that electrostatic repulsive forces help keep whey proteins from aggregating whereby the smaller aggregates provide enhanced stability in solution. Calcium in ALA-1 may also restrict the ability for ALA to form aggregates thereby limiting the size of these aggregates also. There have also been reports where the presence of calcium was found to stabilize the disulphide bond in ALA which enhances its thermal stability (Chang & Li, 2001; Livney, Verespej, & Dalglish, 2003). In the absence of aggregates and near isoelectric conditions (*i.e.* ALA-3 at pH 5.00), the open structure, thermal denaturation and reduced electrostatic repulsion are all factors which promote the formation of large aggregates.

3.4. Interfacial properties

The interfacial tension between canola oil and a 0.1% w/w ALA-1 and ALA-3 solution in response to temperature pre-treatments and solvent pH were measured using a Du Noüy ring method (Fig. 5). Findings were analysed using a three-way ANOVA to find all main factors and the 2-way interaction between temperature and protein-type to be significant ($p < 0.001$) (Table 1). ALA-1 and ALA-3 were found to greatly reduce the interfacial tension of the water-canola oil interface from ~28 mN/m to ~14 mN/m. A Pearson correlation determined that the interfacial properties of ALA were significantly related to the surface charge of these proteins ($r = 0.62$ $p < 0.001$). In all cases, interfacial tension was lower at pH 5.00 than at pH 7.00 due to its proximity to its isoelectric point where it carried less of a net charge allowing for better adsorption at the interface and protein–protein interactions to form a viscoelastic film. ALA-3 proteins were found to be temperature independent whereas ALA-1 experienced slight improvement in their ability to lower interfacial tension when exposed to higher temperatures (Fig. 5). However this trend was only minor.

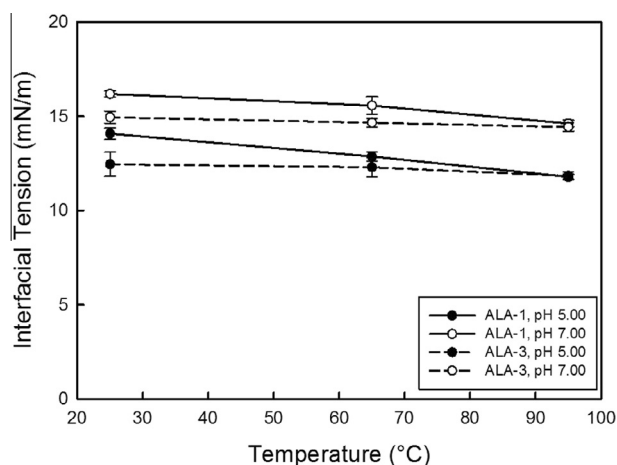


Fig. 5. Mean interfacial tension (mN/m) of ALA-1 and ALA-3 proteins as a function of pH and temperature pre-treatments. Data represent the mean $\pm$ one standard deviation ($n = 3$).

3.5. Emulsifying properties

The emulsion activity index (EAI) (Fig. 6A) and emulsion stability index (ESI) (Fig. 6B) in response to pH and temperature pre-treatments was obtained using a turbidimetric technique developed by Pearce and Kinsella (1978) for dilute protein-stabilized emulsions. A 3-way ANOVA determined that for EAI, the main factors of pH and protein-type, and the two way interactions of: pH $\times$ temperature and pH $\times$ protein-type were significant ($p < 0.001$) (Table 1). EAI measures how well a protein can coat an oil droplet surface (Pearce & Kinsella, 1978). Overall, it was found that ALA proteins at pH 7.00 (regardless of the type) coated the droplets better than those at pH 5.00; however ALA-3 experienced a much larger magnitude difference. For instance, ALA-3 increased from ~71.82 m²/g at pH 5.00 to ~209.2 m²/g at pH 7.00 (difference of ~137.4 m²/g), whereas ALA-1 increased from ~59.22 m²/g at pH 5.00 to ~113.9 m²/g at pH 7.00 (difference of ~54.68 m²/g) (Fig. 6A). ALA-3 is thought to be more flexible and open than ALA-1 due to the absence of stabilizing calcium ions possibly allowing it to reorient at the oil–water interface with greater ease. Furthermore, less protein aggregation was evident (reduced hydrodynamic radius) at pH 7.00 than pH 5.00 due to charge repulsion possibly leading proteins being more spread out along the interface. The interaction of pH $\times$ temperature in EAI values revealed a decrease in the magnitude difference between pH values as temperatures increased, most likely associated with increased hydrophobicity as the proteins start to unravel at higher temperatures. For instance, EAI values increased at 25 °C from

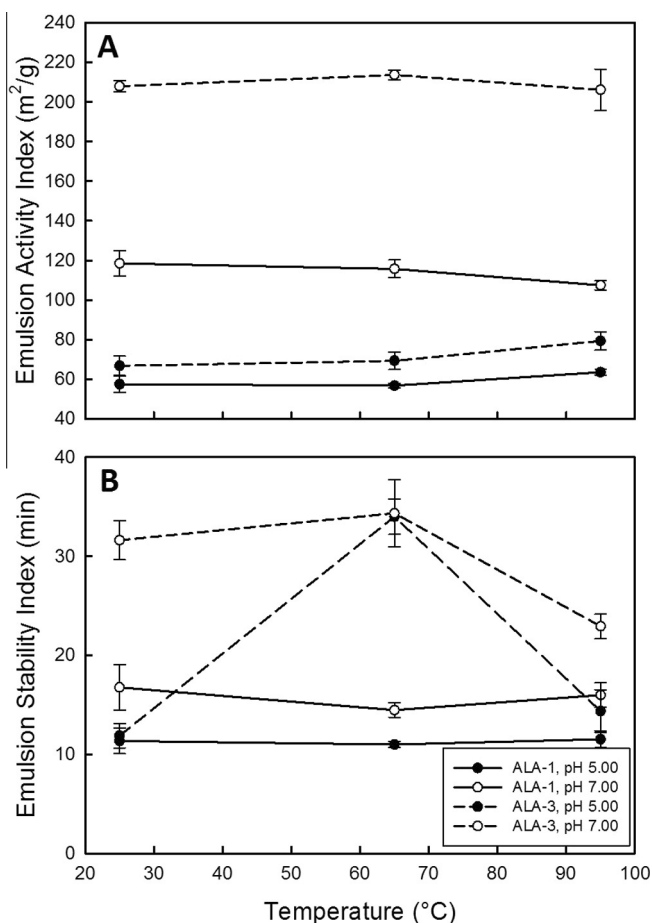


Fig. 6. Mean emulsification activity (m²/g) (A) and stability (min) (B) indices for ALA-1 and ALA-3 proteins as a function of pH and temperature pre-treatments. Data represent the mean $\pm$ one standard deviation ($n = 3$).

65.06 m²/g at pH 5.00 to ~163.2 m²/g at pH 7.00 (difference of ~101.2 m²/g); at 65 °C from 63.03 m²/g at pH 5.00 to ~164.7 m²/g at pH 7.00 (difference of ~101.7 m²/g); and at 95 °C from 71.46 m²/g at pH 5.00 to ~156.8 m²/g at pH 7.00 (difference of ~85.32 m²/g) (Fig. 6A).

The way which proteins coat an oil droplet surface are driven by its physical properties. Pearson correlation coefficients determined the relative significance between EAI and the physical properties of ALA: hydrodynamic radius ($r = -0.34$, $p < 0.05$), surface hydrophobicity ($r = 0.83$, $p < 0.001$), surface charge ($r = -0.73$, $p < 0.001$) and interfacial tension ($r = 0.57$, $p < 0.001$). With respect to hydrodynamic radius of these ALA aggregates, this relatively weak correlation is attributed to changes in tertiary conformation at the interface. Their ability to reorient and adhere to the interface may depend on the relative amounts of disulphide bonds among ALA proteins. Those with many inter-protein disulphide bonds may be less capable of reorienting itself at the interface which prohibits an increase in EAI. Yet for the other physical properties (*i.e.* surface hydrophobicity, surface charge and interfacial tension), the strong significant correlation between them demonstrates the importance of controlling these parameters to enhance droplet coverage. For interfacial tension and surface charge, greater droplet coverage a higher interfacial tension, and a greater surface charge were found at pH 7.00. This suggests that better droplet coverage is not dependent on a neutrally charged protein probably due to electrostatic repulsive charges which may help ALA obtain a more open structure. Electrostatic repulsion may help proteins to spread out across a droplet surface which enhances its droplet coverage. Furthermore, electrostatic repulsive forces which keep proteins from aggregation may be more important in aiding in droplet coverage as opposed to a better adsorption to the droplet surface with a lower interfacial tension. Electrostatic repulsive forces may aid in an evenly coated droplet surface due to their limited ability to cause proteins to form aggregates. With respect to surface hydrophobicity, the strong correlation between these two properties is attributed to the ability for adherence to the interface where higher surface hydrophobicity promotes adsorption.

The ESI values for ALA-1 and ALA-3 in response to pH and temperature are given in Fig. 6B, and analysed using a 3-way ANOVA to find all main factors and subsequent interactions to be significant ($p < 0.001$) (Table 1). ESI values for ALA-3 proteins were highly pH and temperature dependent. At pH 7.00, ESI values increased slightly from ~31.62 min at 25 °C to ~34.34 min at 65 °C, and then declined substantially to ~22.92 min at 95 °C. Whereas at pH 5.00, ESI values greatly increased from ~11.88 min at 25 °C to ~33.99 min at 65 °C, and then declined substantially to ~14.36 min at 95 °C (Fig. 6B). The peak in ESI values at 65 °C was thought to be associated with partial unravelling of the ALA-3 structure and possibly a more open structure. With the exception of 65 °C in which ESI values were similar, ESI was higher at pH 7.00 than pH 5.00 at both 25 °C and 95 °C (Fig. 6B). At low temperatures (*i.e.* 25 °C) the native of ALA may shield many of the charged groups that were successively revealed by unfolding at 65 °C resulting in increased emulsion stability. At 95 °C, the denaturation of ALA results in proteins that is more likely to form aggregates when cooled back to room temperature.

In the case of ALA-1, ESI values were relatively independent of temperature however were found to be higher at pH 7.00 (~15.74 min) than at pH 5.00 (~11.29 min) (Fig. 6B). The lack of temperature dependence may be associated with a more stable tertiary structure due to the presence of calcium ions, which restricts unraveling. Furthermore, the higher net charge at pH 7.00 would create increased electrostatic charge repulsion between the films surrounding oil droplets leading to the formation of more stable emulsions, than at pH 5.00 where films would

have significantly reduced net charges since the proteins are near their pI values.

Though Pearson correlations revealed that EAI was correlated with the physical properties of ALA, ESI was found to correlate to two properties investigated in this study: EAI ($r = 0.66$, $p < 0.001$) and surface hydrophobicity ($r = 0.62$, $p < 0.001$). There are several surprising factors with this result as it was hypothesised that electrostatic repulsive forces given by the surface charge of the proteins would have had an effect on the stability of these emulsions. Furthermore, since surface hydrophobicity and the droplet coverage were found to be significant, it was predicted that the interfacial tension would also play a role in stabilizing an emulsion. Therefore this suggests that the stability of the emulsion had more to do with the ability for these proteins to adhere to the interface as opposed to electrostatically keep droplets from flocculating.

Overall, the electrostatic repulsive forces due to the highly electronegative charge associated with ALA at pH 7.00 enhanced the emulsion stability of ALA. Electrostatic repulsion aided in keeping droplets from flocculating however was less effective for neutrally charged ALA at pH 5.00. Furthermore, increased stability found with ALA-3 compared to ALA-1 is attributed to the more open and flexible conformation of ALA-3 which gave greater ease for the reorientation at the interface. Yet this does not fully explain for the enhanced stability observed with ALA-3 when treated at 65 °C. It was found that there were similar trends observed in ESI (Fig. 6B) when compared to the relative amounts of secondary structure as measured by circular dichroism (Fig. 1). The secondary structure and emulsion stability of ALA-1 was found to be relatively independent of the temperature pre-treatments. In ALA-3, the secondary structure and emulsion stability was found to be dependent on temperature pre-treatments such that an increase in structure and stability was observed when increasing temperature pre-treatments from 25 to 65 °C followed by a decrease in structure and stability when increasing temperatures from 65 to 95 °C. There appears to be a relationship between these two properties which may be explained by how secondary structure may affect the process by which a protein stabilizes a droplet surface. When there are less secondary structures present, it is hypothesised that there are a greater amounts of random loops and coils are present to cause proteins to become more entangled. Proteins may require a greater amount of conformational change as a more entangled protein to optimise its ability to stretch out and adhere to the interface. As proteins adhere to an interface, an entangled protein may be limited in its ability to change in conformation which may yield a sub-optimal conformation at the interface which reduces its emulsion stability. The greater amount of secondary structure reduces the amount of conformational change a protein may undergo which such that less unfolding is needed to adhere to the interface.

4. Conclusions

The effect of pH and temperature pre-treatments on the ALA proteins impacted both their physicochemical and emulsifying properties to varying degrees. Changes in temperature altered the structure of the proteins which then affected its physicochemical properties by increasing their hydrophobicity especially under fully denatured temperatures (*i.e.* 95 °C); and caused changes in the surface charge (*i.e.* from 25 to 65 °C). Both of which influenced their aggregation behaviour, especially ALA-3 at pH 5.00. The pH of the system also influenced the surface charge on both ALA-1 and ALA-3 where enhance electrostatic repulsion at pH 7.00 was observed, resulting in reduced aggregation despite having a greater amount of its hydrophobic moieties at the surface. Findings

indicate that changes to protein conformation using various pH and temperature pre-treatments influences their surface chemistry, aggregation and ability to align at the oil–water interface. Conformational stability in the presence of calcium ions (i.e., ALA-1) also played an important role in ALA performance, where it was found the both ALA-1 and ALA-3 differed considerably. ALA-1 was more resistant to conformational changes in response to pH and temperature pre-treatments than ALA-3. Overall, the absence of calcium ALA-3 was able to reduce interfacial tension more than ALA-1 where calcium stabilized the protein structure and restricted its ability to align and re-orient as well at the interface. As a result, higher droplet surface coverage (EAI) was obtained for ALA-3 than ALA-1. The increased ability of ALA-3 to adhere to the interface also allowed for greater emulsion stability. Overall, emulsions were more stable for ALA-1 and ALA-3 when at pH 7.00 than pH 5.00 due to the presence of electrostatic repulsive forces between protein coated droplets. Furthermore, a temperature pre-treatment of 65 °C on ALA-3 resulted in an increase in its secondary structure resulting the greatest emulsion stability presumed due to the partially denaturation and refolding at this temperature. The greater amount of secondary structure allows for a less entangled protein which may enhance its ability to unfold to enhance emulsion stability. This demonstrates that it is possible to tailor the properties of the protein by adjusting the main factors (i.e. pH, temperature and protein-type) to obtain desired functionality. Under the conditions examined in this study, ALA-3 pre-treated at 65 °C and at pH 7.00 resulted in the best emulsifying properties.

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