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EFFECT OF ENZYMATIC LIPOPHILIZATION ON THE FUNCTIONAL PROPERTIES OF BOVINE α-LACTALBUMIN

EFFECTO DE LA LIPOFILIZACIÓN ENZIMÁTICA SOBRE LAS PROPIEDADES FUNCIONALES DE LA α-LACTOALBÚMINA BOVINA

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Abstract

Bovine α-lactalbumin (α-LA) was enzymatically modified with a lipase from *Rhizopus orizae* in an organic environment which catalyzed the covalent bonding of different length chain fatty acids, at reaction times of 3 and 7 days. Structural changes and functional properties of native and enzymatically modified α-LA in a pH range from 3 to 10 were analyzed. The degree of modification was higher than 40 %. The enzymatic lipophilization modified the functionality of the α-LA due to the increase in the surface hydrophobicity. In general, the surface properties were improved by the lipophilization specially at alkaline pH values, increasing the emulsifying activity more than 60 % and showing higher foam stability.
Keywords: lipophilization, lipase, α-lactalbumin, functional properties, foaming.

Resumen

La α-lactoalbúmina (α-LA) bovina se modificó enzimáticamente utilizando una lipasa de *Rhizopus orizae* en medio orgánico, la cual catalizó la unión covalente de ácidos grasos de diferente longitud de cadena, probándose tiempos de reacción de entre 3 y 7 días. Se estudiaron los cambios de estructura, propiedades funcionales de la α-LA nativa y modificada en un intervalo de pH 3 a 10. Se obtuvieron rendimientos de reacción por encima del 40%. La lipofilización causó cambios en la funcionalidad de la α-LA, que se atribuyeron a un aumento en la hidrofobicidad superficial. En general las propiedades superficiales mejoraron por la lipofilización especialmente a valores de pH básicos, aumentando la actividad emulsionante más del 60 % y mostrando una mayor estabilidad de la espuma.
Palabras clave: lipofilización, lipasa, α-lactoalbúmina, propiedades funcionales, capacidad espumante.

1 Introduction

Proteins are being used increasingly in foodstuffs to promote functional properties like solubility, gelation, emulsification, and foaming (Panyam and Kilara, 1996). The modifications of proteins can be carried out to improve or provide some functional properties (Vidal *et al*., 2003). Lipophilization is a technique to induce such modifications via the insertion of hydrophobic residues, such as fatty acids, in a protein. This can be done in mostly three different ways (Akita and Nikai, 1990; Hacke *et al*., 1982; Nakai *et al*.,

1980; Watanabe *et al*., 1981). The main lipohilization techniques include thermal treatments, and enzymatic and chemical lipophilization, (Panyam and Kilara, 1996). However, the enzymatic modification technique has important advantages over the others to modify proteins due to its mild reaction conditions, specificity, and minimal collateral reactions and by-products, so it can improve the functional properties of proteins without the production of undesirable secondary products produced by less specific lipophilization techniques (Chobert *et al*., 1996, Villeneuve, 2007; Wada *et al*., 2001). The modification catalyzed by enzymes in different substrates has gained increasing

attention on account of its diversification in industrial applications such as the transformation of fats and oils, the production of surfactants, the racemic resolution of compounds, the biodiesel production, and the lipophilization of sugars, phenolic compounds and proteins (Stergiou *et al.* 2013; Villeneuve, 2007). Lipases are preferred to catalyze the lipophilization reactions because they are stable and versatile and able to hydrolyze triacylglycerols and synthetize esters by esterification and transesterification (Correa-Leyva *et al.*, 2017; Reetz *et al.*, 1997; Sun *et al.*, 2013; Wada *et al.*, 2001).

Lipases and esterases can be isolated from different fungi in solid state fermentations (Camacho-Ruiz *et al.*, 2016, Ramos-Ibarra *et al.*, 2017). Roussel *et al.* (1997) modified soy protein isolates with a lipase from *Rhizopus arrhizus* to bind fatty acids of different length chain with a yield of 50 %. The use of enzymes from GRAS fungi such as *Rhizopus orizae* has been highly recommended by the FDA (Cantabrana *et al.*, 2015). This enzyme has been widely used for esterification processes (Ghamgui *et al.*, 2004) but there is not enough information available on the functionality of the enzymatic lipophilized proteins. So, this work is focused on the study of the changes in structural and functional properties of an enzymatically lipophilized bovine α-lactalbumin.

2 Materials and methods

2.1 Materials

Bio PURE^{TM} α-lactalbumin (α-LA) from bovine milk was supplied by Davisco Foods International (Eden Prairie, MN, USA). The product was isolated from fresh, sweet dairy whey. Protein was fully soluble in a wide pH range. It contained 6.5 % max. of moisture, 90.0 %min of protein, 1.0 % max of fat, 3.5 % of ash and 0.2 % of lactose according to the supplier.

Corn oil (Mazola^{TM}, Mexico) used in the present research was purchased in a local supermarket (Mexico City, Mexico). Lauric and stearic acids and the fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS) were purchased in Sigma Aldrich (St. Louis, USA).

2.2 Sample preparation

Native and enzymatically lipophilized α-LA were stored in hermetically sealed containers under refrigeration (4 °C) until used. All the analyses were performed by dissolving the α-LA protein (native or modified) for 30 min with magnetic agitation at room temperature (∼ 25 °C) in 0.1 M phosphate buffer at different pH values (3, 5, 7, and 10).

2.3 Methods

2.3.1 Enzymatic lipophilization

The enzymatic lipophilization was performed according to the methodology proposed by Roussel *et al.* (1997) with some modifications. α-LA (10 g), enzyme (3 g), and the fatty acid (30 g) were placed into 250 ml flasks. *Tert*-butyl alcohol (50 ml) was then added and the flask hermetically sealed and the mixture incubated at 35°C in an incubator with orbital agitation (150 rpm). The protein / free fatty acid ratio was 1/3 (w/w) and protein / enzyme ratio was 10/3 (w/w). The fatty acids tested were lauric and stearic. The enzyme used was a lipase from *Rhizopus orizae* (10U/mg) and the hydrophobic medium was provided by *tert*-butilic alcohol which is fully inert to the lipase action. Reaction times of 3 and 7 days were tested. The free fatty acids that did not react were removed with hexane. The yield of lipophilized protein was determined by the difference between the initial free fatty acids added and the final free fatty acids extracted with hexane.

2.4 Structure assays

2.4.1 Degree of modification (DM)

DM was determined by the *ortho*-phthaldialdehyde (OPA) assay as described by Matemu *et al.*, (2011). An aliquot of 50 μL of a 0.1 % sample was mixed with 1 mL of OPA reagent and incubated at room temperature for 2 min. Optical density of the sample was read at 340 nm (Geneysis 10S UV-Vis, USA). DM was calculated as the difference in the absorbance of the acylated and unmodified samples.

2.4.2 Circular dichroism (CD)

CD was used to evaluate conformational changes in the secondary structure of the proteins (Rodiles-López *et al.*, 2010) in a range of 180 to 250 nm in a Jasco J-715 spectropolarimeter (Jasco Inc., Easton, MD, USA) equipped with a PTV-348WI peltier-type cell holder for temperature control. Protein solutions of 0.1 mg/mL were prepared in 5 mM phosphate buffer (pH values of 3, 5, 7, and 10) in 0.10 cm cells. Results were

expressed as molar ellipticity (degree cm^2/dmol). The secondary structure estimation was assessed according to Pérez-Iratxeta and Andrade-Navarro (2008).

2.4.3 Surface hydrophobicity

Surface hydrophobicity was determined using the hydrophobic fluorescence probe 1-anilino-8-naphtalensulfonate (ANS) (Nakai and Kato, 1980). Samples were prepared at a 1 μM concentration in 5 mM phosphate buffers. A 1:100 protein/ANS (w/w) mixture was prepared. The interaction with ANS was analyzed with an ISS k2 spectrofluorometer (ISS Inc., Champaing, IL, USA) equipped with a water jacketed cell holder for temperature control. The ANS excitation was performed at 380 nm and emission spectra was measured from 400 to 600 nm at 25 °C. both, the excitation and emission bandwidths were set at 1 nm.

2.4.4 Intrinsic fluorescence

The intrinsic fluorescence spectra were measured spectrofluorometrically (Edwin and Jagannadham, 1998). Briefly, a 1 μM protein sample was dispersed in 5 mM phosphate buffer (pH 7). The excitation wavelength was of 280 nm and the emission was measured over a range of 300-450 nm (slit width1 nm).

2.4.5 ζ potential

Experiments were performed as described by Arroyo-Maya *et al.* (2012). Zeta potential was measured in 1:10 (v/v) diluted samples in deionized water over a pH range of 3 to 10 at 25 °C in a Malvern Zetasizer Nano S (model MAL1600, Canada) immediately after their preparation.

2.5 Functional properties evaluation

2.5.1 Solubility index (SI)

SI was determined according to the technique of Bera and Mukjerkee (1989) with slight modifications. Diluted protein solutions (1%, v/v) in 0.1 M phosphate buffers adjusted to pH values of 3, 5, 7, and 10, were stirred (30 min) and centrifuged (10,000 x g for 20 min at 20 °C). The protein content in the supernatant was determined by the Bradford method (1976). The SI was calculated with Eq. (1):

$$SI(\%) = \frac{\text{Protein in the supernatant}}{\text{Protein in the sample}} \times 100 \quad (1)$$

2.5.2 Emulsifying activity index (EAI)

The EAI was determined according to Pearce and Kinsella (1978). A mixture of 30 mL of 0.5% protein solution in 0.1 M phosphate buffer adjusted at different pH values and 10 ml of corn oil was prepared. Each mixture was homogenized for 60 s with a blender (D 130 Wiggen Hauser, Sdn Bhd). Fifty millilitres of emulsion were dispersed into 5 mL of 0.1 % sodium dodecyl sulphate (SDS). Absorbance was measured at 500 nm with a spectrophotometer. The EIA was calculated using eqs (2)-(3):

$$T_b = \frac{2.303A}{I} \quad (2)$$

$$EAI = \frac{2T_b}{\O c} \quad (3)$$

Where A is the absorbance of the sample at 500 nm; I the length of the cell; $\O$ the volume of the sample (mL) and c the concentration of protein (%).

2.5.3 Emulsion stability

The emulsion stability was determined with a Turbiscan Lab Expert optical analyzer (Expert, Formulation Inc., France). The principle of the measurement was based on the variations of the droplet volume fraction (migration) or diameter (coalescence) resulting in a variation of back scattering (BS) and transmittance signals (Lemarchand *et al.*, 2003). Briefly, 18 mL of sample were transferred to a cylindrical glass cell. The emulsion destabilization was analyzed with the BS profiles with a light beam of 880 nm at different time intervals. The variations of the BS signal (ΔBS) was calculated as the difference between BS at the initial and final times. Finally, using the Turbiscan Lab Expert software, the Turbiscan Stability Index (TSI) was calculated to estimate the stability of the emulsion (Wisniewska, 2010) using Eq. (4):

$$TSI = \sqrt{\frac{\sum_{i=1}^{n} x_i - x_{BS}}{n - 1}} \quad (4)$$

Where x_i is the backscattering value for each time measured; x_{BS} is the average of x_i and n is the number of measurements.

Emulsions were prepared in a 3:1 protein dispersion ratio; both the native and the modified proteins were previously dispersed in 0.1 M phosphate buffer at different pH values. Emulsions were monitored each 10 min up to an hour in the equipment.

2.5.4 Foaming capacity (FC) and foaming stability (FS)

The FC and FS were evaluated according to Puski (1975) with slight modifications. A 1 % (w/v) protein dispersion was blended for 90 s at 12,000 rpm and the foaming capacity was calculated with the Eq. (5):

$$CFE = \frac{(B - A)}{A} \times 100 \qquad (5)$$

Where A is the initial volume and B the final volume after blending.

For FS, the foam volume was registered after 30 min of sample rest and was calculated with Eq. (6) (Haque *et al.*, 1982):

$$FS = \frac{F^2}{F^1} \times 100 \qquad (6)$$

Where F^1 is the foam volume after agitation and F^2 is the foam volume after 30 min rest.

2.5.5 Surface and interfacial tension

The surface and interfacial tension of the native and lipophilized proteins were estimated according to Daverey and Pakshijaran (2010). Briefly, a surface tensiometer (DCAT 11, Dataphysics Instruments, Germany) with the Wilhelmy plate method was used The Wilhelmy plate was 10 mm in length, 19.9 mm in width and 0.2 mm height. Several 0.25 % (w/v) protein solutions were prepared in 0.1 M phosphate buffer (pH 7) at 20°C and their surface tension measured against corn oil.

2.6 Statistical analysis

All data were representative of at least 3 separate experiments. These results represent the means ± standard deviations of triplicate determinations. A one-way analysis of variance was used to evaluate the differences between the native and lipophilized samples using $\alpha = 0.05$.

3 Results and discussion

3.1 Structure properties

3.1.1 Reaction yield and degree of modification

The reaction yield obtained for the enzymatic lipophilization of α-LA with lauric and stearic acids catalyzed by the lipase from *Rhizopus oriyzae* was higher than 40 % for all the enzymatical lipophilization reactions (Table 1). This result was similar to the one previously reported by Roussel *et al.* (1997) who obtained 50 % of reaction yield in a lipophilization catalyzed by lipase S in the presence of *tert*-butanol with a mixture of palmitic and stearic acid in soy protein. Meanwhile, Haque *et al.* (1982) reported a reaction yield between 53 and 77% depending on the incorporation degree of fatty acids when soy glycinine was chemically lipophilized. Better results were obtained when non-protein substrates were lipophilizated. Lopez *et al.* (2007) modified chlorogenic acid with an immobilized lipase obtained from *Candida antartica* with different chain length alcohols obtaining a product yield between 61 and 93 % by the chemical-enzymatic way and between 40 to 60 % by the direct enzymatic way.

The reaction yield in the esterification reaction catalyzed by lipases may be affected by diverse factors such as the organic solvent chosen, the nature and accessibility of the substrate, the temperature, the water activity and the pH of the microenvironment of the enzyme (Stergiou *et al.*, 2013). The use of different enzymes for the lipophilization of amino acids reported evident changes in the reaction yield for the previous factors indicated (Godtfredsen and Bjoerkling, 1990; Nagao and Kito, 1989; Zhang *et al.*, 2005).

In the enzymatic lipophilization, the lipase catalyzes the nucleophilic attack of the -NH$_2$ of the amino acid on the carboxyl group of the fatty acid. Thus, in the case of α-LA there are 12 lysine residues where the lipohilization reaction might occur. However, the free amino residues in a protein are not the only incorporation points since other covalent bonds could be formed between the fatty acids and the hydroxyl group of serine, threonine and tyrosine (Roussel *et al.*, 1997). The free amino groups were measured by the OPA method and the α-LA contained 12 possible reactive amino groups. Modified amino groups were inferred from OPA results. All results were reported in relation to the 100 % of amino groups in the native protein. The degree of modification was shown in Table 1.

The degree of modification is significantly different ($p \leq 0.05$) for each fatty acid tested and the reaction time is only significant in the case of the lauric acid probably due to the chain length of the fatty acid and the stabilization time of the reaction (Table 1).

Table 1. Reaction yield and degree of modification of enzymatically lipophilized α-lactalbumin.

	Yield (%)	-NH$_2$ moles modified per molecule	Degree of modification (%)
α-LA-LAU-3	42.48	2.12	82.30 ± 0.34^a
α-LA-LAU-7	41.98	1.44	87.96 ± 0.69^b
α-LA-EST-3	46.06	3.09	74.20 ± 1.38^c
α-LA-EST-7	45.07	2.73	77.18 ± 2.03^c

Results are the mean of three replicates $\pm$ SD. Values in the same column followed by different lowercase letters are significantly different ($p \leq 0.05$). α-LA represents the native α-lactalbumin; LAU represents the lauric acid; EST represents the stearic acid; 3 and 7 represents the reaction time in days.

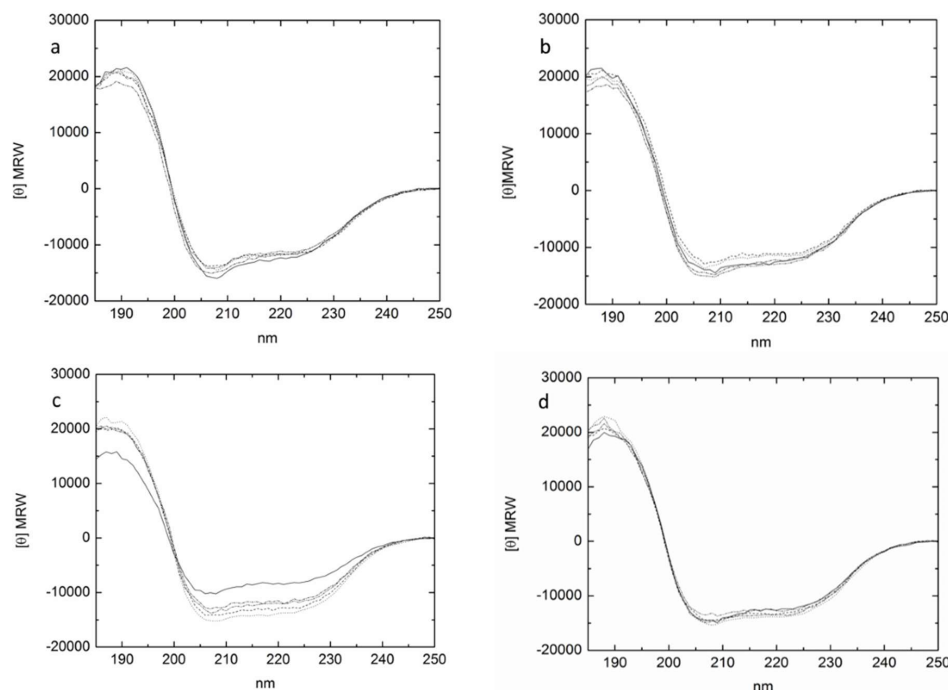Fig 1. Effect of enzymatic lipophilization) on the circular dichroism spectra in the far UV at pH a) 3.0, b) 5.0, c) 7.0, d) 10.0 of α-lactalbumin (α-LA) — α-LA, - - - α-LA-LAU-3, ·· ·· α-LA-LAU-7, -·- α-LA-EST-3, ·--·· α-LA-EST-7

Roussel *et al.* (1997) observed the maximum incorporation of the fatty acid after 5 days of reaction independently of the concentration of lipase S used, when soy protein was lipophilized.

Results obtained here indicated that between 12.04 and 25.8 % of available amino groups were substituted in α-LA with the fatty acids. The lauric acid modified α-LA showed 82.30 and 87.96 % of free amino groups for reaction times after 3 and 7 days, respectively. The modified α-LA with stearic acid showed the highest degree of modification with 74.20 and 77.18 % of free amino groups for reaction times after 3 and 7 days indicating that the length of the chain of the fatty acid plays an important role in the degree of modification. Similar results were reported by Matemu *et al.*, (2012).

They found that longer fatty acid chains reacted better than shorter fatty acid chains when peptides of soy proteins were chemically modified.

3.1.2 Circular dichroism (CD)

CD can measure changes in the secondary structure of α-LA in response to the enzymatic lipophilization and pH. CD spectra in the far ultraviolet are shown in Figure 1. The spectra of the protein showed the two characteristic peaks for the peptide bond at 190 and 208 nm. The native α-LA had a negative ellipticity in the far UV spectra at 208 nm (Kuwajima *et al.*, 1985). In the dichroism spectra at pH 3.0 (Figure 1a) a slight increase in the medium residue molar ellipticity of the modified α-LA with respect to the native α-

LA ([θ] MRW) was observed; at pH 7 there was a little negative decrease in the molar ellipticity in the 3 days reaction as well as in the 7 days reaction (α-LA-LAU-7). However, at pH 10, (Figure 1d) the changes were more evident for the α-LA modified with stearic acid after 7 days of reaction (α-LA-EST-7). These changes in the ellipticity may be due to modifications in the charge of some amino acids which induce the breaking of electrostatic interactions and/or the generation of new interactions with neighboring amino acids (Robles-López *et al.*, 2012).

According to the CD spectra, the process of enzymatic lipophilization of α-LA induced changes in its secondary structure. This is contrary to a previous report by Akita and Nakai (1990) in which they did not find significant differences in the structure of chemically liophilized β-lactoglobulin with stearic

acid and the native protein. Meanwhile, Robles-López *et al.* (2012) found some changes when α-LA was physically modified with pulsed electric fields.

The experimental CD spectra were evaluated using the K2D2 software (Pérez-Iratxeta and Andrade-Navarro, 2008). The α-helix, β-strand, and random structure contents in native and modified α-LAs by enzymatic lipophilization at pH values of 3, 5, 7, and 10 are shown in Table 2. The results in this study are in agreement with a previous study for the protein structure (Robbins and Holmes, 1970) which reports 25-26% α-helix, 14-15% β-strand, and 60% random structure. In the case of the native α-LA, no changes in secondary structure were observed at the pH values of 5, 7, and 10, however, an important increase in α helix content was recorded at pH 3 where the molten globule state of the molecule is present.

Table 2. Percentages of α-helix, β-sheet and random structure in enzymatically lipophilized α-LA at different pH values

	α-helix (%)	β-sheet (%)	Random structure (%)
pH 3			
α-LA	35	15	50
α-LA-LAU-3	35	16	49
α-LA-LAU-7	36	16	49
α-LA-EST-3	28	15	57
α-LA-EST-7	35	16	49
pH 5			
α-LA	29	14	57
α-LA-LAU-3	31	15	53
α-LA-LAU-7	27	15	58
α-LA-EST-3	29	14	57
α-LA-EST-7	30	14	56
pH 7			
α-LA	28	14	59
α-LA-LAU-3	31	14	55
α-LA-LAU-7	34	14	53
α-LA-EST-3	29	15	56
α-LA-EST-7	28	14	58
pH 10			
α-LA	29	14	57
α-LA-LAU-3	31	14	55
α-LA-LAU-7	33	14	53
α-LA-EST-3	30	14	56
α-LA-EST-7	28	14	57

α-LA= native α-lactalbumin; LAU = Lauric acid; EST = Stearic acid at 3 and 7 reaction time in days

Significant increases of up to 25% in the α-helix content of the enzymatically lipophilized α-LAs was observed. A similar behavior was reported by Aouzelleg *et al.* (2004) in the α-helix content of β-lactoblobulin when the intensity of high hydrostatic pressures (HHP) and temperature treatments increased. The most evident changes in the enzymatic lipophilization treatment occurred in the case of α-LA modified with lauric acid after 7 days of reaction (α-LAU-7). Changes in the secondary structure depended on pH, the incorporated fatty acid and the reaction time.

3.1.3 Surface hydrophobicity

The results of the surface hydrophobicity, as measured by extrinsic fluorescence, of the enzymatically lipophilized α-LA are shown in table 3. The extrinsic fluorescence intensity increased slightly as a consequence of the lipophilization; this fact may indicate that the protein tendency to bind the ANS probe increased with the treatment. This may be due to a deployment of the hydrophobic sites of α-LA toward the aqueous phase after the lipophilization (Shilpashree *et al.*, 2017). Other authors have found an increase in surface hydrophobicity in modified proteins by physical (Robles-López *et al.*, 2012) and chemical (Akita and Nakai, 1990) methods.

The highest values of fluorescence were recorded in the case of the stearic acid-modified α-LA with increases of 1.9, 1.6, 1.45 y 1.2 times for α-LA-EST-3 at pH values of 3, 5, 7 and 10 respectively compared with the native protein (α-LA, pH 7). The highest hydrophobicity was found in α-LA-EST-3 at pH 3 with 2.67. It was also observed that the higher the pH, the smaller the hydrophobicity. The increase in hydrophobicity at pH 3 indicates a higher exposition of the hydrophobic surface at this pH value. As previously indicated, at pH 3 the α-LA shows a molten globule state which is a compressed and transitional folding state amidst the native and unfolded states, characterized by a conserved secondary structure and the exposition of a great number of hydrophobic groups (Lala and Kaul, 1992; Yang *et al.*, 2006). These results agree with those reported by Rodiles-López *et al.* (2010) where the α-LA was physically modified with HHP finding an intensity of fluorescence at pH 3 almost 20 times higher than at other pH values.

In general, no changes were observed in the maximum emission wavelength register of extrinsic fluorescence in the enzymatically lipophilized α-LA in the presence of ANS at different pH values with respect to the native α-LA (pH 7) in this study. An exception to this was observed in the cases of α-LA-EST-3 at pH 5, and α-LA-EST-3 and α-LA-EST-7 at pH 3 which showed a blueshift of 11 nm with respect to the native α-LA (pH 7).

3.1.4 Intrinsic fluorescence

The effect of enzymatic lipophilization over the α-LA structure at different pH values was studied also through intrinsic fluorescence. The increase of fluorescence intensity by the incorporation of the different fatty acids to the native α-LA may be an indication of a decrease in the polarity of the tryptophan environment caused by the displacement of the water molecules by the binding of the non-polar molecules (Muresan *et al.*, 2001). A decrease in the fluorescence intensity was also observed by Rodiles-López *et al.* 2010 after modificating the α-LA by physical methods and they attributed it to the reduced unfolding of the modified protein. In this study, the change in the fluorescence intensity was dependant on the chain length of the incorporated fatty acid that could be related to a higher exposition of tryptophan residues (Table 3). The fluorescence intensity was higher for the lipophilized α-LA with stearic acid at pH values of 7 and 10. These samples showed a significant increase (~ 100 %) in the fluorescence intensity compared with the native α-LA. In the case of the molten globule state (pH 3), the mean increase in fluorescence could be due to the reorganization of the tryptophan residues in the molten globule conformation, with a concomitant alteration of the solvent accessibility to several tryptophan residues and release of quenching by adjacent amino acids and the incorporated fatty acid chains (Chaudhuri *et al.*, 2010). The α-LA fluorescence spectra at pH 7 showed the maximum emission wavelenght at 315 nm which may indicate that at this pH value the tryptophan residues are buried inside the protein. However, with the decrease of the pH, the maximum emission wavelenght changed. At pH 3 the maximum emission wavelenght of fluorescence was 322 nm indicating the presence of tryptophan residues on the protein surface (Wu *et al.*, 1998). Again, a blueshift in the maximum emission wavelength could be observed for the α-LA-EST-3 and α-LA-EST-7 samples.

Table 3. Effect of enzymatic lipophilization on the hydrophobicity of α-lactalbumin measured by extrinsic fluorescence (IF) and intrinsic fluorescence (EF) at different pH values.

		pH 3		pH 5		pH 7		pH 10	
		λ_{max*}	NIF**	λ_{max*}	NIF**	λ_{max*}	NIF**	λ_{max*}	NIF**
α-LA	EF	482	1.29	483	1.02	484	1	483	1.09
	IF	322	0.14	316	0.21	315	1	316	0.93
α-LA-LAU-3	EF	481	0.87	482	1.07	484	0	485	0.98
	IF	318	0.87	315	0.69	315	0.92	316	1.24
α-LA-LAU-7	EF	484	0.82	483	0.92	483	1.03	484	1.07
	IF	320	0.86	314	0.74	314	0.96	315	1.19
α-LA-EST-3	EF	473	2.67	478	1.74	484	1.46	484	1.26
	IF	324	1.25	318	1.06	306	1.68	322	2
α-LA-EST-7	EF	473	1.89	483	1.63	484	1.45	484	1.21
	IF	315	1.57	318	1.28	315	2.27	315	1.75

α-LA= native α–lactalbumin; LAU= lauric acid; EST= stearic acid; 3 and 7 is the reaction time in days *λ_{max} = maximum emission wavelength (nm) of intrinsic/extrinsic fluorescence. ** NIF = normalized fluorescence intensity compared to α-LA pH 7 (value 82237 arbitrary units of fluorescence for intrinsic fluorescence; 72854 arbitrary units of fluorescence for extrinsic fluorescence).
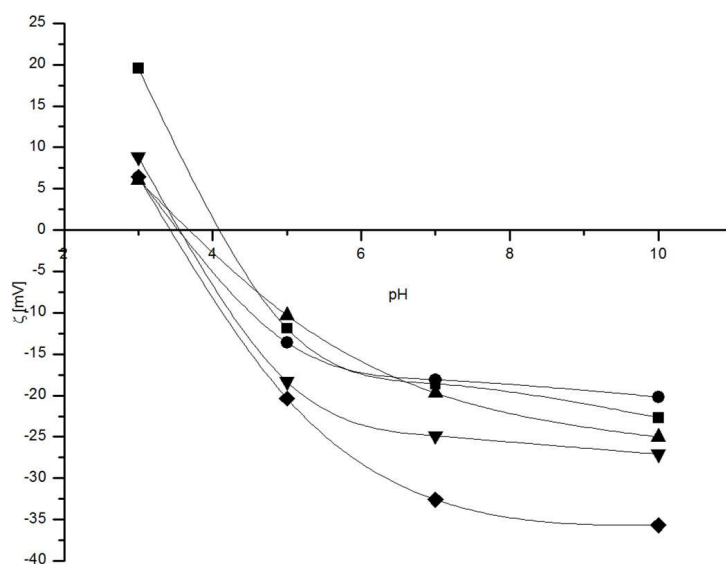

Fig 2. Effect of enzymatic lipophilization on the net charge of native and enzymatically modified α-lactalbumin (α-LA) at pH from 2.0 to 10.0. ■ α-LA, · α-LA-LAU-3, ▲ α-LA-LAU-7, ▼ α-LA-EST-3, ♦ α-LA-EST-7.

3.1.5 ζ potential

Figure 2 shows the net charge of the native and the enzymatically lipophilized α-LA samples as a function of pH. It can be observed that the ζ potential decreased when the pH increased. The enzymatic lipophilization increased the negative net charge in α-LA possibly due to conformation changes or to the reorganization of the protein molecule. The reorganized lipophilized protein

molecules could be exposing some negatively charged groups at basic pH values increasing the negativity of the modified molecule. Additionally, the reduction in positive charges could also possible occur when the positively charged lysine α-amino groups reacted with the carboxyl groups of the fatty acid through a lipase catalized amidation reaction (Conde *et al.*, 1999).

The isoelectric point of the native α-LA was 4.1, close to previously reported by Bramaud *et al.* (1997)

for native α-LA (between 4.2 and 4.6). The isoelectric points for the α-LA-LAU-3, α-LA-LAU-7, α-LA-EST-3, and α-LA-EST-7 decreased to 3.55, 3.70, 3.55, and 3.45, respectively.

3.2 Functional properties

3.2.1 Solubility

Solubility is a good functionality index of proteins because it is a practical measurement of the protein denaturation and aggregation (Jambrak *et al.*, 2010). Table 4 shows the solubility values of enzymatically lipophilized α-LA at four pH values. It could be observed that enzymatic lipophilization caused an important solubility loss compared with the native protein that may be related to the increase of hydrophobicity in the protein molecule (Aoki *et al.*, 1981). The solubility decrease was nearly 38.75 % depending on the incorporated fatty acid, the reaction time and the pH. Similar results were found by Akita and Nakai (1990) as the amount of fatty acids chemically-attached to β-lactoglobulin increased. Similarly, Roussel *et al.* (1997) observed that the solubility of enzymatically lipophilized soy protein decreased with respect to the native protein.

The highest values of solubility in this research were found at alkaline pH values and the lowest at acid pH values. A drastic solubility loss has been reported by Rodiles-López *et al.* (2008) at values close to the isoelectric point when the α-LA was physically modified by HHP. The protein-protein interaction increases near the isoelectric point because the electrostatic forces are weaker and less water is interacting with the protein molecules reducing the solubility (Pelegrine and Gasparetto, 2005). The lowest solubility value was observed in enzymatically lipophilized α-LA with stearic acid after a 7-day reaction time.

3.2.2 Emulsifying activity index (EAI)

Enzymatic lipophilization of α-LA improved its emulsifying activity (Table 4). This improvement may indicate that the protein modification induced an unfolding of the protein chains and thus the exposition of the hydrophobic section (Akita and Nakai, 1990; Feeney *et al.*, 1982). The EAI of α-LA improved more than 60 % with the incorporation of the fatty acids. This improvement has been previously reported by several authors (Akita and Nakai, 1990; Chobert *et al.*, 1989; Kito, 1987; Matemu *et al.*, 2011; Sen *et al.*, 1981; Wong *et al.*, 2006) who
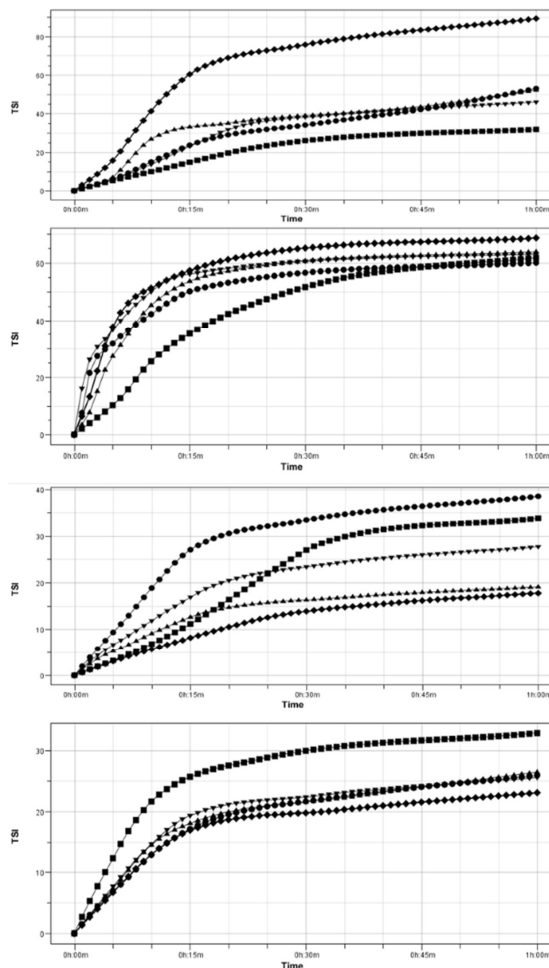

Fig 3. Stability kinetics of 3:1 protein-corn oil (v/v) emulsions of enzymatically modified α-lactoalbumin (α-LA) with different fatty acids at pH values of a) pH 3, b) pH 5, c) pH 7, d) pH 10. ■ α-LA, · α-LA-LAU-3, ▲ α-LA-LAU-7, ▼ α-LA-EST-3, ♦ α-LA-EST-7.

considered that this phenomenon is derived from the increase in the amphipathic nature of the proteins due to the modification.

The highest EAI values for enzymatically modified α-LA were found at pH 7 and 10. These results indicate a significant improvement in the emulsifying activity at alkaline pH values, and are in opposition to those reported by Rodiles-López *et al.* (2008) who found better results at pH 3. Moreover, they pointed out that the MGs at low pH are good emulsifying agents when α-LA were modified by HHP. In this case, the longer the chain of the incorporated fatty acid, the higher the EAI of the enzymatically modified α-LA after a 7-day reaction time.

Table 4. Effect of fatty acid incorporation on the solubility (S), emulsifying activity index (EAI), and Turbiscan stability index (TSI) of native and enzymatically lipophilized α-lactalbumin at different pH values (3, 5, 7, and 10).

	pH 3			pH 5			pH 7			pH 10		
	S (%)	EAI (m²g⁻¹)	TSI	S (%)	EAI (m²g⁻¹)	TSI	S (%)	EAI (m²g⁻¹)	TSI	S (%)	EAI (m²g⁻¹)	TSI
α-LA	96.47± 0.25aB	110.60± 11.65Aa	31.8	74.78± 0.86bA	78.27± 2.13BA	61.8	93.29± 8.28adA	101.79± 0.39aA	33.8	98.86± 0.54cdA	71.88± 10.22bcA	32.8
α-LA-LAU-3	75.04± 0.93aB	112.35± 5.15ab	53	76.82± 0.32aA	89.63± 1.84bB	60.1	77.01± 0.18bB	123.87± 2.74cB	38.5	77.44± 0.28cbBC	178.80± 6.86dBD	25.9
α-LA-LAU-7	71.06± 0.13aC	99.36± 3.08aa	52.2	72.10± 0.56bB	91.16± 7.52acAC	63.9	71.69± 0.24cbC	108.48± 7.75aA	19.1	71.54± 0.45abBC	175.25± 8.00bCB	26.4
α-LA-EST-3	69.30± 0.34aD	91.99± 0.37aad	46	70.45± 1.22aC	87.97± 1.53bCB	63.1	71.30± 1.96aC	123.99± 2.03cC	27.7	65.62± 13.91aC	181.06± 1.76dDC	25.6
α-LA-EST-7	60.55± 1.15aE	111.61± 8.73ac	89.3	64.04± 1.13bD	88.61± 4.38bDCB	68.7	63.41± 0.53cbdD	138.27± 0.33cD	17.8	68.24± 4.17dbD	185.07± 1.81dEB	23.1

*Results are the mean of three replicates ± SD. Values in the same row followed by different lowercase letters are significantly different ($p \leq 0.05$); values in the same column followed by different capital letters are significantly different ($p \leq 0.05$). *alpha*-LA= native α-lactalbumin; LAU= lauric acid; EST = stearic acid; 3 and 7 = reaction time in days.

However, Wong *et al*. (2006) did not consider that the emulsifying properties were related to the lenght of the incorporated fatty acids chain after they chemically lipophilized casein.

3.2.3 Emulsion stability

Several 3:1 protein:corn oil emulsions were studied to observe the effect of the enzymatic lipophilization of the α-LA on the emulsions stability. In Figure 3 the stability kinetics of these emulsions at different pH values are shown after 3 and 7 days of reaction time. The kinetics of stability showed an improvement in the emulsion stability in the case of the enzymatically lipophilized samples at alkaline pH values. Roussel *et al*. (1997) reported extremely stable emulsions (more than 8 months at 45°C) when soy protein was enzymatically lipophilized. The emulsion stability at pH 7 from highest to lowest was α-LA-EST-7, α-LA-LAU-7, α-LA-EST-3, α-LA-LAU-3 and at pH 10 α-LA-EST-7, α-LA-EST-3, α-LA-LAU-3, α-LA-LAU-7.

The EAI at pH 3, 5, 7, and 10 after 60 minutes for native and enzymatically modified α-LA with lauric and stearic acid are shown on Table 4. The results obtained in the present research showed an improvement in the protein with the highest incorportation degree, namely the α-LA enzymatically lipophilized with stearic acid after of 7 days of reaction time. These results are in opposition to those found by Akita and Nakai (1990) with chemically lipophilized β-lactoglobulin where the emulsion stability decreased in the proteins with a higher degree of fatty acid incorporation. This behavior could be related to an excessive denaturalization of proteins (Aoki, *et al*., 1981). In general, the ability of α-LA to create and stabilize emulsions was influenced by the enzymatic binding of hydrophobic chains (Matemu *et al*., 2012).

3.2.4 Foaming capacity (FC) and foam stability (FS)

The hydrophobic amino acid residues form hydrophobic regions which are adsorbed in the air-water interface of proteins. However, in most proteins, these hydrophobic regions are relatively protected and any factor increasing the exposition to the hydrophobic regions of the proteins is able to increase the surface activity of the protein (Murray, 2007). The effect of enzymatic lipophilization in α-LA on ita FC is shown in Figure 4.
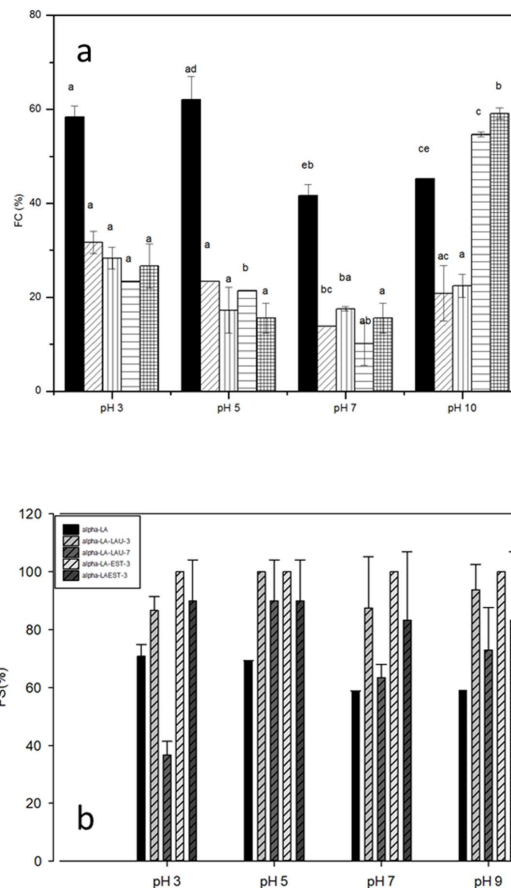

Fig 4. Effect of enzymatic lipophilization and pH on a) foaming capacity (FC) and b) foam stability (FS) of α-lactalbumin (α-LA.).

The enzymatically modified α-LA showed an increase of up to 29 % compared with the native α-LA in FC (Figure 4a). The highest values of FC were found in the α-LA modified with stearic acid after a 7-day reaction time. The high values of FC found in the present research are opposed to those previously reported by Akita and Nakai (1990) who only found a slightly increase in FC in modified proteins with low fatty acid incorporation; moreover, a high fatty acid incorporation gave even lower FC. These results, however, are in agreement with those reported by Haque *et al*. (1982) who found an increase in FC in palmitoyl glycinine (soybean) caused by the amphipathic nature of the modified protein. In a similar way, Rodiles-López *et al*. (2008) found an important increase in the FC of physically modified α-LA with HHP which produced partially deployed structures that contribute to the adequeate flexibility around the air-liquid interface.

Table 5. Effect of enzymatic lipophilization on the surface and interfacial tension of α-lactalbumin in 0.1M phosphate buffer at pH 7 and 20 °C.

	Surface tension (mNm^{-1})	Interfacial tension (mNm^{-1})
α-**LA**	48.235 ± 0.163a	11.095 ± 0.025a
α-**LA-LAU-3**	31.414 ± 0.027b	11.353 ± 0.029a
α-**LA-LAU-7**	30.996 ± 0.025cb	12.001 ± 0.022a
α-**LA-EST-3**	40.415 ± 0.030d	10.956 ± 0.024ab
α-**LA-EST-7**	39.872 ± 0.029ed	10.771 ± 0.010ac

Results are the mean of three replicates ± SD. Values in the same column followed by different letters are significantly different ($p \leq 0.05$). α-LA= native α-lactalbumin; LAU = lauric acid; EST = stearic acid; 3 and 7 = reaction time in days.

Although the enzymatic modification of the protein improved both the FC and FS, the mechanism is different due to the fact that FC and FS need different molecular characteristics of the foaming agent. The FC needs a rapid adsorption of proteins in air-water interface during the bubble and the ability to undergo a rapid conformational change and rearrangement at the interface level. Moreover, FS needs a thick, cohesive, continuous, air-permeable protein film around each gas bubble (Fennema, 1985).

An increase in the foam stability also could be observed as a consequence of the fatty acid incorporation (Figure 4b). This is in opposition to the data reported by Wong *et al.*, (2006) who found a decrease in the foam stability when the glycomacropeptide of κ-casein was modified with lauric and myristic acids. This decrease was considered a result of the decrease in solubility and of the reduction in accessibility to the air-water interface.

The FS of enzymatically lipophilized α-LA is shown in Figure 4. The α-LA-EST-3 showed the highest stability at all the pH values tested and α-LA-LAU-3 was more stable at pH 5. When the pH values were close to the isoelectric point of the native protein, the FS of the lipophilized protein was higher than that of the native protein, probably due to the increase in the protein-protein interactions and to the intramolecular cohesion, which are factors with the capability to reduce the surface tension.

The increase in the foam stability could be due to the protein denaturation and the increase in hydrophobicity. Protein denaturation provides a higher stability to the foam since it forms a more cohesive structure which enhances the protein-protein interaction which is a necessary factor for foam stability (Kinsella, 1976, Yasumatsu *et al*. 1972). On the other side, when hydrophobicity increases, the interfacial tension decreases. This improves the stability because the hydrophobic region of proteins increases its resistance to migration to the aqueous phase (Haque and Kito, 1983; Keshavarz and Nakai, 1979; Nakai, 1983).

3.2.5 Surface and interfacial tension

The hidrophobicty has been related to an improvement in the foam formation (Kato and Nakai, 1980; Moro *et al.*, 2001). Once the interface contacts are done, the natural flexibility inside the molecules can expose hydrophobic portions, previously buried, to the interface, potentially leading to interfacial denaturation of the molecules (Graham and Phillips, 1979). The obvious result is the reduction in surface tension. The values of surface tension for the enzymatically lipophilized α-LA at different pH values are shown on Table 5. The surface tension of α-LA was 48.23 mNm^{-1}, close to a surface tension of 45 mNm^{-1} reported for various protein solutions (Prins *et al.*, 1998). Surface tension significantly decreased (p$\leq$ 0.05) with the lipophilization from 48.23 to 30.99 mNm^{-1} (α-LA-LAU-7). So, the lipophilization may reduce the surface free energy in the air-protein solution interface. The highest capacity to reduce the interfacial tension was shown in the modified α-LA with lauric acid at 3 days of reaction time. Lowest values of surface tension were obtained with α-LA-EST-3. No significant changes were observed with the reaction time, but with the incorporated fatty acid. A more important factor than the surface tension is the protein capacity to quickly reduce the surface tension which has been related with an improved foamability (Wilde *et al.*, 1996). This may explain the higher foam formation and stability of the modified α-LA with stearic acid, although this did not show the lowest surface tension values.

The interfacial surface values for the different protein solutions and corn oil are also shown on Table 5. Results show a slight decrease in the interfacial tension in the water-corn oil interface from 11.095 to 10.771 mNm^{-1} as a result of the lipophilization process. The capability of a protein to decrease the interfacial tension is related to an increase in its EAI due to the changes in hydrophobicity and in conformation caused by the lipophilization. This may explain the improved capacity of these samples to adsorb in the water-corn oil interface (Chobert *et al.* 1988). This is in agreement with the findings of Nakai *et al.* (1990) who found a correlation between surface hydrophobicity and high emulsifying activity. On the other hand, Keshavarz and Nakai (1979) observed that the higher the hydrophobicity of a protein, the lower the interfacial tension and the higher the emulsifying activity; hydrophobicity is higher when more fatty acids are bonded.

Conclusions

The preparation of fatty acid esters of α-LA through a reaction catalyzed by a lipase from *Rhizopus orizae* in organic medium was feasible with a good yield. These modifications in the native α-LA changed the protein structure, increased the surface hydrophobicity, and reduced the solubility enhancing in this way its functional properties. The emulsifying and foaming properties increased with the length of the fatty acid chain incorporated. The best results were obtained in the case of the α-LA enzymatically modified with stearic acid and suspended in an alkaline pH buffer.

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