O/W emulsions stabilized by whey protein: Influence of heat treatment and high pressure homogenization

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ABSTRACT

The objective of this work was to study the physical properties of O/W emulsions stabilized by whey proteins. Coarse emulsions were prepared with 30% (w/w) soy oil and water phase containing 7% w/w of non heated and denatured whey proteins at 70 or 90ºC. These emulsions were high pressure homogenized at 250, 450 and 600 bar in order to obtain fine emulsions. Creaming kinetics of the emulsions obtained after and before high pressure homogenization was measured. At equilibrium conditions of phase separation it was also evaluated the droplet size distribution and flow curves of the separated phases. The effect of high-pressure homogenization on the protein denaturation degree were evaluated by polyacrylamide gel electrophoresis (PAGE) and associated with emulsion properties. A cream phase separation was observed after 1 day of coarse emulsions prepared with non heated and treated at 70ºC and after 15 days for 90ºC heat denatured proteins. After pressure homogenization all emulsions were stable with no phase separation even after 30 days of observation. Fine emulsions showed the smallest droplet size and Newtonian flow behaviour with non heated protein in contrast to all emulsions containing heat denatured proteins that showed shear-thinning, thixotropic behavior and gel formation. The SDS PAGE of non-heat treated emulsions showed whey proteins unfolding and some aggregation as a consequence of high-pressure homogenization. It was observed the presence of high molecular weight protein aggregates stabilized for disulfide bonds and concentrated into the cream phase, mainly in fine emulsions homogenized at 250 bar. However, the increase in pressure homogenization caused the disruption of these aggregates, particularly of β-lactoglobulin. The decrease in the particle size in fine emulsions homogenized at 450 bar was related to disruption of these aggregates.

Keywords: whey proteins; food emulsions, thermal denaturation, phase separation; high pressure homogenization.

INTRODUCTION

A wide variety of food products can be integrally or in part constituted by emulsions like milk, yogurt, salad dressings, mayonnaise, ice cream and many others. Emulsions are thermodynamically unstable, which can show phase separation. However, emulsions can show kinetics stability with the addition of emulsifiers during the homogenization process [1].

Whey proteins can be used as emulsifiers due to its excellent surface activity, which can avoid the coalescence or cremeation process of the dispersed phase, giving stability to the oil/water (O/W) interface [2]. The main fractions of whey proteins are β-lactoglobulin (β-Lg), α-lactoalbumin (α-La) and bovine serum albumin (BSA). Native form the whey proteins show a rigid and compact structure that are stabilized by intermolecular interactions. Under heating, whey proteins show unfolding of these molecular structures, leading to an interaction of the reactive sites like thiol groups (-SH) and resulting in intermolecular disulphide bonds (S-S) and aggregation of the protein fractions. Partial denaturation of whey proteins can increase their functional properties and application as food ingredients. In food emulsions, a partial denaturation of whey proteins can contribute to the formation of a thicker coating of the oil-water interface, contributing to the increase in emulsion stability. In this way mixture of non heated and thermal denatured whey proteins has been used to act as emulsifying agent, since native proteins move more quickly to the oil/water interface facilitating the covering, while denatured proteins contribute for the formation of thick membranes on this interface [3].

The main fraction of whey proteins corresponds to β-Lg, which denatures if heated by at least 30 minutes at a temperature around of 60ºC. However, when β-Lg dispersions are heated during the same time but at temperatures around 95ºC, it is expected a complete protein unfolding, which is attributed to a

conformational transition and the extensive exposure of hydrophobic groups and highly reactive nucleophilic sites (-SH and ε-NH^{+3}).

The high pressure homogenization process of emulsions can produce emulsions with smallest droplet size, increasing the interaction between the interface (O/W) and the protein used as emulsifier. However, such process can unexpectedly produce unstable emulsions. This fact can be attributed to the coalescence of the dispersed phase due to the interaction of the small droplets as a consequence of high pressure leading to aggregation of unfolded proteins [4]. Lee and co-workers [5] also found that homogenization at high pressures (500 to 2000 bar) of emulsions (10% w/w soybean oil and 0.5% w/w whey proteins) resulted in partial denaturation of protein adsorbed at the (O/W) interface with the exposure of its hydrophobic groups in a similar way to that observed in heating processes. Thus, the improved adsorption of proteins at the interface, caused by high pressure homogenization contributed to the stability of the system.

The aim of this study was to evaluate the effect of different heat treatment conditions on emulsion containing whey proteins through stability analysis during storage and rheological properties. In addition, the influence of high pressure homogenization on such properties of emulsions was also evaluated.

MATERIALS & METHODS

Whey protein isolated (WPI) was donated by Arla Foods Ingredients (Denmark), soybean oil was obtained from local market and all other reagents were purchased from Sigma Aldrich Co. (St. Louis, USA). 10% (w/w) whey protein stock solutions were prepared by powder dissolution in deionized water under mild magnetic stirring at room temperature and heat treatment of these solutions were performed under agitation at 70 or 90ºC by 30 minutes. After heat treatment, protein solutions were immediately cooled using an ice bath. Emulsions were prepared using heated denatured whey protein solutions as well non heated proteins.

Coarse emulsions were firstly prepared by homogenizing the soybean oil in the aqueous phase with a homogenizer Ultra Turrax model T18 (IKA, Germany) for 5 minutes at 14000 rpm. For all coarse emulsions, the whey protein and oil content were fixed at 7% (w/w) and 30% (w/w), respectively. Sodium azide (0.01%, w/w) was added to prevent microbial growth. Fine emulsions were prepared by high pressure homogenization of coarse emulsions in a double step homogenizer Panda 2K NS1001L (Niro Soavi, Italy) operating pressure values ranging between 250 to 600 bar at the first step and 50 bar at the second step. Emulsions were homogenized and were collected in a container (first cycle). Part of this emulsion was then subjected to a new cycle of homogenization under the same pressure conditions (second cycle). Then, the effect of emulsion homogenization under one or two cycles of high pressure treatment was also evaluated.

All emulsions were evaluated considering their stability. Evaluation of emulsions stability was determined immediately after emulsion preparation. 10 mL of each emulsion was poured into a cylindrical glass tube sealed with a plastic cap and stored at 25ºC for a period necessary to obtain phase separation equilibrium. The emulsion stability was expressed in terms of the volume of the separated phase and the time necessary to reach the phase separation equilibrium. Separated phases on stable emulsions were subjected to optical microscopy and rheological evaluation. Emulsions samples were poured into microscope slides, covered with glass cover slips and observed using a conventional optical microscope Carl Zeiss Model mf-AKS 24 x 36 EXPOMET (Zeiss, Germany). 40x and 100x objective lenses were used to visualize the microstructure of coarse and fine emulsions. The microscopic images of the coarse emulsions were analysed with the public domain software Image J v1.36b (http://rsb.info.nih.gov/ij/). Micrographs were transformed into 8-bit grey scale binary images of 640–480 pixels and were then segmented by 'thresholding'. The pixel-scale values were converted into microns by a scaling factor using methodology described by Perrechil and Cunha [7]. This process of image analysis to determine the droplet size was carried out only for coarse emulsions, while for fine emulsions the diameter was obtained with laser diffraction measurements. A Mastersizer S (Malvern Instruments Ltd., UK) was used to determine the average diameter of the fine emulsion droplets. The size of the oil droplets was also determined as the volume–surface mean diameters (d_{32}) [7]. Rheological measurements of the emulsions were performed with a stress-controlled rheometer (Carri-Med CSL2 500, TA Instruments, England) using a double walled concentric cylinder consisting of an inner rotating acrylic cylinder (inner radius = 20.38 mm, outer radius = 21.96 mm) and an outer fixed stainless steel cup (inner radius = 20 mm, outer radius =22.38 mm). Flow curves were obtained by an up-down-up steps program using different shear stresses range for each sample, in order to give a maximum shear rate value of 300 s^{-1}.

Protein aggregation under high pressure homogenization was evaluated by SDS page under reducing and non-reducing conditions. For this purpose fine emulsions were subjected to centrifugation in order to accelerate phase separation processes, since no phase separation was observed for these emulsions during

experiments of stability. PAGE experiments were performed only for non heated proteins since emulsions formulated with heat denatured whey proteins exhibited gelation after high pressure homogenization, which did not allow the phase separation. Polyacrylamide gel electrophoresis (PAGE) was done using 5% and 15% polyacrylamide stacking and resolution gel, respectively. Reduced conditions was determined by adding samples at a concentration of 10 mg mL^{-1}, in the loading buffer containing Tris-HCl with β-mercaptoethanol and SDS, while non reducing conditions the loading buffer contained only Tris-HCl and SDS. These mixtures were boiled for 5 min at 95ºC after the loading in polyacrilamide gel. Native pure fraction of whey proteins and commercial pre stained Invitrogen™ Bench Marker protein leader (Byoagency International, Jacksonville, USA) were used as markers. Fine emulsions that did not exhibit gelation after high pressure homogenization were centrifuged in order to accelerate phase separation. Serum phases were then evaluated by a polyacrylamide gel electrophoresis (PAGE) using reducing and non reducing (SDS PAGE) conditions [6] in order to evaluate the effect of high-pressure homogenization on the protein denaturation degree.
Significant differences between the droplet sizes and rheological parameters were determined by the Tukey test. Statistical analyses were performed using the software STATISTICA 6.0 (Statsoft Inc., Tulsa, USA) and the level of confidence was 95%.

RESULTS & DISCUSSION

A cream phase separation (5 ml) was observed after 1 day of coarse emulsions preparation for systems stabilized with non heated and treated at 70ºC, but only after 15 days of storage for emulsions containing heat denatured proteins at 90ºC. After pressure homogenization all emulsions were stable with no phase separation even after 30 days of observation. Fine emulsions formulated with non heated whey proteins showed thinner consistency (liquid like, with a milky appearance). However, fine emulsions formulated with denatured proteins (particularly at 90ºC) showed gelation after high pressure homogenization, especially after the second cycle of homogenization.
Typical microstructure of coarse and fine emulsions can be seen in Figure 1. All coarse emulsions (Fig. 1 a to c) showed individual droplets evenly distributed, which showed greater diameters for non heated proteins (21.7±1.0 μm). The temperature increase from 70ºC to 90ºC led to significant decrease of droplets size from 17.8±2.2 μm to 12.2±1.3 μm, respectively. Such decrease in droplet size could be associated with the exposition of hydrophobic groups of whey proteins that easily interact with oil phase, stabilizing the O/W interface and avoiding or delaying the cremeation process [3].
The high pressure homogenization led to a significant decrease of droplets size for emulsions with non heated proteins (Fig. 1-d), in a similar way to the pattern observed for emulsions containing heat denatured proteins at 70ºC and homogenized under 250 bar (data not shown). Such droplets reduction contributed to the increase in emulsions stability during storage.
The increase of pressure homogenization (Fig 1-e) or the addition of 90ºC heat denatured proteins (Fig. 1-f) led to formation of clusters of aggregated particles. They could be great proteins aggregates eventually with oil droplets in their interior, which could also be related to the increase of viscosity or gelation of these systems.
Table 1 shows the mean droplet diameter (d_{32} - μm) of fine emulsions. Fine emulsions containing non heated proteins showed smaller droplet diameter. Moreover, in general, the increase of heat treatment temperature associated with the increase of pressure homogenization caused a significant increase in droplets size. In addition two cycles of homogenization at higher pressures caused an increase of droplet size of fine emulsions prepared with heat denatured proteins.

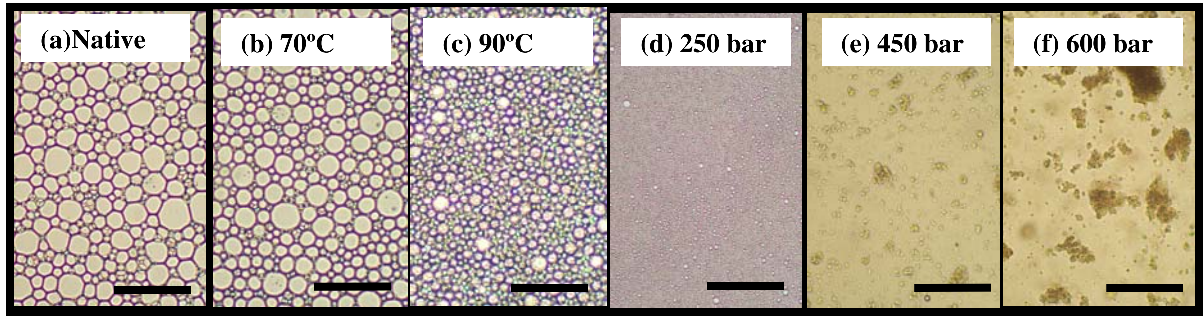

Figure 1. Microstructure of emulsions containing 30% (w/w) soybean oil and 7% (w/w) whey proteins. Coarse emulsions with non heated proteins (a) or heat denatured at 70 (b) or 90ºC (c). Fine emulsions with proteins heat-treated at 70ºC and homogenized at 250 (d) and 450 bar (e). Fine emulsions with proteins heat-treated at 90ºC and homogenized 600 bar (f). Scale bar = 100 μm

Table 1: Mean droplet diameter (d_{32} - μm) of fine emulsions

Homogenization pressure	Homogenization cycle	d_{32} (μm)		
		Non heated	**70°C**	**90°C**
250	**1**	1.28^{abA} (± 0.02)	2.19^{aB} (± 0.05)	4.18^{aC} (± 0.31)
	2	0.98^{bA} (± 0.07)	2.23^{aB} (± 0.12)	4.81^{bC} (± 0.27)
450	**1**	0.61^{cA} (± 0.05)	3.77^{bB} (± 0.23)	6.55^{cC} (± 0.49)
	2	1.45^{aA} (± 0.30)	3.33^{bB} (± 0.45)	6.75^{cdC} (± 0.42)
600	**1**	1.88^{dA} (± 0.11)	6.77^{cB} (± 0.21)	4.23^{aD} (± 0.17)
	2	1.22^{abA} (± 0.50)	9.32^{dB} (± 1.11)	7.04^{dC} (± 0.28)

Different small letters = significant differences ($p < 0.05$) in the same column and different capital letters indicate significant differences ($p < 0.05$) in the same line.

Figure 2 (A) shows the typical flow curves observed for the cream phase of coarse emulsions, while Figure 2 (B) shows the typical flow curves of fine emulsions prepared with heated denatured proteins. The serum phase of coarse emulsions as well as fine emulsions prepared with non heated whey proteins showed Newtonian flow behaviour (data not shown). Such results could be correlated to the minor amount of oil in serum phase or smaller droplets of fine emulsions, respectively.

Cream phase of coarse emulsions (Fig 2 – A) showed greater values of shear stress at the second ramp (increase of shear rate), which could indicate an unexpected rheopectic flow behaviour. Such condition could be attributed to an increase of dispersed phase droplets collisions during flow, which could cause an aggregation of these droplets [1].

On the other hand, fine emulsions prepared with heat denatured proteins showed a pronounced thixotropic and shear thinning behaviour (Fig. 2 – B) that probably corresponded to the structure formed by the large aggregates observed for those systems (Fig. 1- e and f). On the other hand, the shear thinning behaviour is the most expected for food emulsions (second ramp up of shear stress in Fig 2 –B) and in such a case, it could be related to several reasons as the alignment of dispersed phase spherical droplets or serum exudation during shear with possible disruption of emulsion structure [8].

The values of Newtonian viscosity of serum phase of coarse emulsions increased as the temperature of heat treatment was higher. It ranged from 2.5 ± 0.3 mPa.s for non heated proteins to 3.3 ± 0.1 mPa.s and 10.7 ± 0.5 mPa.s for treated at 70 and 90ºC, respectively. However, the effect of heat treatment on whey proteins was less pronounced on apparent viscosity (at shear rate of 100 s^{-1}) of the cream phase of coarse emulsions.

Table 2 shows the Newtonian viscosity of fine emulsions containing non heated whey proteins, as well as the apparent viscosity of the other fine emulsions, measured at shear rate of 100 s^{-1} (η_{100} - Pa s). This rheological parameter was evaluated at this shear rate because it is typical for food processes such as flow through a pipe, stirring or mastication [7].

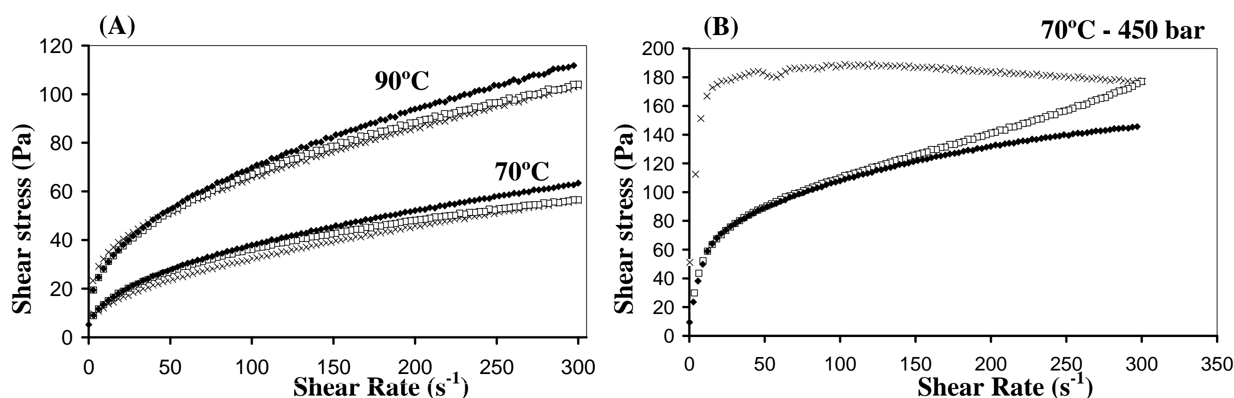

Figure 2. Effect of different process conditions on rheological properties of emulsions stabilized by whey proteins. Flow curves of: (A) cream phase of coarse emulsions prepared with heated whey proteins at 70 or 90ºC, and (B) fine emulsion prepared with heated whey proteins at 70 ºC and high pressure homogenised at 450 bar. Shear rate steps are (╳) first ramp-up rate (☐) ramp down rate (◆) second ramp-up rate

Table 2: Effect of different process conditions on Newtonian or apparent viscosity η_{100} (Pa s) at 100 s^{-1} of fine emulsions

Homogenization pressure	Homogenization cycle	Newtonian viscosity (mPa.s)	Apparent Viscosity η_{100} (Pa.s)	
		Non heated	70°C	90°C
250	1	4.46^{a} (± 0.05)	0.039^{a} (± 0.003)	0.553^{a} (± 0.011)
	2	8.40^{be} (± 0.47)	0.196^{a} (± 0.010)	1.187^{ab} (± 0.047)
450	1	5.85^{c} (± 0.04)	1.151^{b} (± 0.082)	1.434^{b} (± 0.061)
	2	7.82^{b} (± 0.13)	3.122^{c} (± 0.119)	3.613^{c} (± 0.227)
600	1	6.78^{d} (± 0.12)	0.600^{d} (± 0.020)	1.677^{b} (± 0.011)
	2	9.13^{e} (± 0.38)	1.585^{e} (± 0.015)	4.558^{d} (± 0.427)

Different small letters = significant differences ($p < 0.05$) in the same column and different capital letters indicate significant differences ($p < 0.05$) in the same line.

The fine emulsions showed greater values of viscosity as the temperature of protein denaturation increased. Similar effect was observed concerning pressure homogenization as well as the second cycle of pressure.

The relation between high pressure homogenization and pressure cycles on protein denaturation was evaluated by polyacrylamide gel electrophoresis experiments (Figure 3). It was observed the formation of aggregates with greater molar mass at the top of stacking gel, specially for non heated fine emulsions homogenized at 250 bar (Fig 3- A). The increase of pressure homogenization caused the decrease of such top bands (Figure 3 A and C) with the increase of corresponding bands of pure whey protein fractions (middle part of the PAGE gels). It was also observed that the addition of β-mercaptoethanol caused accentuated disruption of such aggregates (Fig, 3 B and D), which indicate that these aggregates were mainly stabilized by internal disulphide bonds formed during previous protein heat treatment as well as during the high pressure homogenization process.

The PAGE results suggests that above 450 bar the high molar mass protein aggregates were disrupted by the high shear forces. High pressure is associated with partial protein unfolding and exposition of hydrophobic sites that contributed to the emulsion stability as commented before. Moreover, the increase of pressure causes a further unfolding on protein, which led to an increase in emulsion viscosity or even the gelation of the fine emulsion.

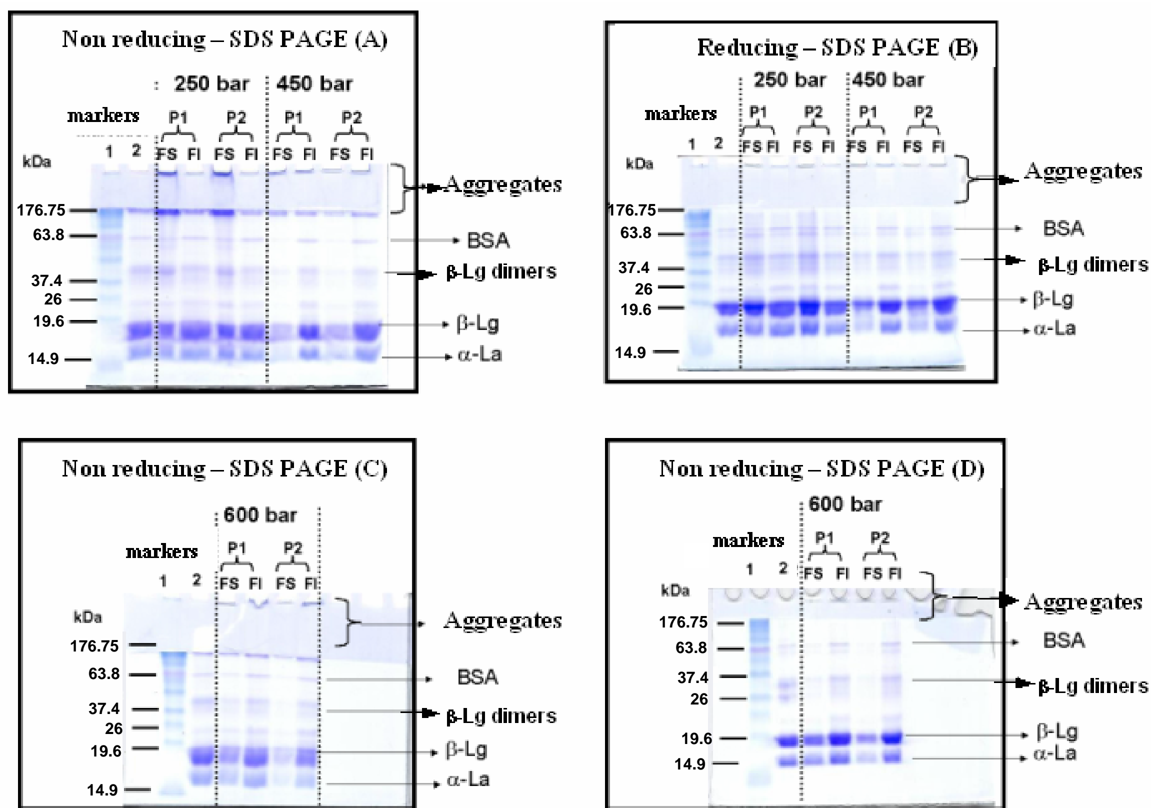

Figure 3. SDS PAGE of emulsions containing native whey proteins. (A and C) Non reducing conditions. (B and D) Reducing conditions. P1 and P2 means first and second homogenization cycle, respectively. FS and FI means top and bottom phase obtained after centrifugation. Commercial marker was loaded at lane 1 and pure whey proteins fractions at lane 2.

CONCLUSION

The results of this work indicated that it is possible to design different whey protein emulsion textures inducing an appropriate degree of whey protein denaturation as well as monitoring the adequate conditions of process homogenization in order to produce desirable structural changes. Such fact can lead to stable emulsions with different attributes that can be used to produce or formulate different food products.

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