Food Hydrocolloids 25 (2011) 398–409

Contents lists available at ScienceDirect

Food Hydrocolloids

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

Freeze-thaw stability of oil-in-water emulsions prepared with native and thermally-denatured soybean isolates

Gonzalo G. Palazolo [a,c,*], Pablo A. Sobral [b], Jorge R. Wagner [a,c]

[a] Laboratorio de Propiedades Funcionales de Alimentos, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Roque Sáenz Peña 352, B1876BXD Bernal, Provincia de Buenos Aires, Argentina
[b] Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CCT La Plata – CONICET), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, Calle 47 (s/n) y 116, B1900AJJ La Plata, Provincia de Buenos Aires, Argentina
[c] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

ARTICLE INFO

Article history:
Received 30 November 2009
Accepted 10 July 2010

Keywords:
Freeze-thawing
O/w emulsions
Soy protein isolates
Stability

ABSTRACT

Freeze-thaw stability of oil-in-water emulsions prepared with native or thermally-denatured soy isolates (NSI and DSI, respectively) as the sole emulsifier and sunflower oil ($\phi = 0.25$) has been examined at various protein concentrations (0.5, 1.0 and 2.0% w/v), comparatively with sodium caseinate (SC). The freeze-thaw stability was assessed by measurements of particle size, oiling off and gravitational separation after isothermal storage at −20 °C for 24 h and further thawing. The oil phase remained in liquid state and the amount of ice formed was similar (>97%) whatever the sample type and protein concentration. At 0.5%, NSI and DSI emulsions where highly unstable, exhibiting a coagulated cream layer with appreciable oiling off (>25%), whereas those prepared with SC were more stable, due to their initial lower flocculation degree (FD %) and particle size. For all emulsions, the increase of protein concentration (0.5–2.0% w/v) improves the freeze-thaw stability as a consequence of a decrease of initial FD %. At 2.0%, where is enough protein to cover the interface, a lower coalescence stability of NSI emulsion respect to those prepared with NSI was observed after freeze-thawing. This result can be attributed to the high tendency to aggregation of native soy globulins at subzero temperatures. Notwithstanding this, unlike the SC emulsions, the formation of new flocs in soy isolates-stabilized emulsions during freeze-thawing cannot be totally controlled.

1. Introduction

Proteins are surface-active molecules that can be used as emulsifiers because to their ability to facilitate the formation, improve the stability and produce desirable physicochemical properties of oil-in-water (o/w) emulsions (McClements, 2004). Among proteins of vegetable origin, soy proteins play an important role in several food systems because of their high nutritional value and functional properties (Kinsella & Whitehead, 1988; Molina, Papadopoulou, & Ledward, 2001). Soy protein isolates are used as emulsifiers in food emulsions due to the surface-active properties of their constitutive proteins, the storage globulins 7S (β-conglycinin) and 11S (glycinin). These protein isolates are generally obtained in non-denatured state from isoelectric precipitation by acidifying an aqueous extract of defatted soy flour, and further solubilization and neutralization of precipitate (Sorgentini & Wagner, 1999; Yamauchi, Yamagishi, & Iwabuchi, 1991).

The functional properties of soy isolates can be modified by subjecting the proteins to various chemical, thermal or enzymatic treatments (Nir, Feldman, Aserin, & Garti, 1994; Qi, Hettiarachchy, & Kalaphaty, 1997). Heating is one of the most important and frequently used methods for denaturing food proteins for processing (Arai & Watanabe, 1988; Kinsella & Whitehead, 1988; Sorgentini, Añón, & Wagner, 2002). It was found that thermal treatment of 7S and 11S globulins induces dissociation, denaturation and aggregation phenomena and these structural modifications of storage globulins are affected by pH, ionic strength and protein concentration (Yamauchi et al., 1991). Therefore, heating of soy isolate proteins also have influence on the stability of o/w emulsions to creaming, coalescence and flocculation in quiescent conditions and under shear stress, as was reported in previous works (Mitidieri & Wagner, 2002; Palazolo, Mitidieri, & Wagner, 2003; Palazolo, Sorgentini, & Wagner, 2004, 2005).

Sodium caseinate is an important dairy ingredient commonly used as an ingredient in a wide range of formulated food dispersions. This sample is composed of a soluble mixture of disordered

* Corresponding author at: Laboratorio de Propiedades Funcionales de Alimentos, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Roque Sáenz Peña 352, B1876BXD Bernal, Provincia de Buenos Aires, Argentina. Tel.: +54 11 43657100x5615; fax: +54 11 43657132.
E-mail address: gpalazolo@unq.edu.ar (G.G. Palazolo).

0268-005X/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodhyd.2010.07.008

hydrophobic proteins having a strong tendency to absorb rapidly at the oil-water interface during emulsification, providing long-term stability to oil-in-water emulsions through a combination of electrostatic and steric stabilization mechanisms (Dickinson, 2006; Dickinson, Golding, & Povey, 1997).

Freezing storage is one of the most important preservation methods for maintaining microbiological and chemical stability and extending the shelf life of food products (Xiong, 1997). There are many potential applications for oil-in-water emulsions that can be frozen and then thawed prior to use, e.g. refrigerated and frozen food or pharmaceutical products (Bamba et al., 1995; McClements, 2004). Nevertheless, most of o/w emulsions are highly unstable when they are frozen and rapidly breakdown after thawing. When an o/w emulsion is cooled, a variety of physicochemical processes can occur including fat crystallization, ice formation, freeze-concentration, interfacial phase transitions and biopolymer conformational changes (McClements, 2004). At present, the stress induced by freezing and the capacity of different ingredient combinations to resist them is poorly understood (Ghosh & Coupland, 2008; Thanasukarn, Pongsawatmanit, & Mc Clements, 2004).

At present, there are few systematic studies of freeze-thaw stability of o/w emulsions prepared with soy isolates as the sole emulsifying agent (Palazolo & Wagner, 2007). Hence, the main objective of this work was to study the freeze-thaw stability of o/w model emulsions prepared with native and thermally-denatured soybean isolates. Sodium caseinate was utilized as emulsifier in o/w emulsions subjected to freeze-thawing (Cramp, Docking, Ghosh, & Coupland, 2004; Ghosh & Coupland, 2008; Ghosh, Cramp, & Coupland, 2006). This protein sample is constituted by proteins which have structural and interfacial properties markedly different respect to those of soy globulins (Tornberg, Olsson, & Persson, 1997). Therefore, comparative study of freeze-thaw stability of emulsions prepared with sodium caseinate was also included in this article.

2. Materials and methods

2.1. Materials

Defatted soy flour (not thermally inactived to avoid protein denaturation) was provided by Solae Latin America (Barueri, SP, Brazil). The soy flour contains 95 g dry solids per 100 g powder and the composition (in dry basis, w/w, as given by the producer) was: 56.0% crude protein (N × 6.25), 7.0% ash, 3.5% total lipids (0.8% triglycerides) and 14.0% dietary fiber. Refined sunflower oil (Molinos Río de la Plata, Argentina) was purchased in a local supermarket. Sodium caseinate (SC) and bovine serum albumin were obtained from Sigma (Sigma–Aldrich Co, St Louis, MO, USA). SC sample was utilized without further purification. The crude protein content of SC (N × 6.38) was 90.4% (w/w). Sudan III (Solvent Red 23, CI number 26100) was purchased from Chroma Gesselschaft (Schmidt GmbH & Co., Stuttgart-Untertükheim, Germany). The chemical reagents used in this work were all of analytical grade.

2.2. Methods

2.2.1. Preparation of native soybean protein isolates (NSI)

Native soy protein isolates (NSI) was prepared according to experimental procedure reported by Sorgentini and Wagner (1999). The defatted soy flour was extracted for 2 h at 20 °C with deionised water (adjusted to pH 8.0 with 2.0 M NaOH); water: flour ratio was 10:1. The mixture was centrifuged at 10,400*g* for 15 min at 20 °C (Beckman Coulter Avanti J25 centrifuge, JA14 rotor, Beckman Coulter, Fullerton, USA). The supernatant was adjusted to pH 4.5 with 1.0 M HCl, then kept for 2 h at 4 °C and subsequently centrifuged at 10,400*g* for 20 min in the same conditions. The precipitate was washed with water, resolubilized in water by neutralization at pH 8.0 with 2.0 M NaOH at room temperature, freeze-dried (Thermovac freeze-dryer, Thermovac Industries Corporation, USA) and ground. NSI was stored as a freeze-dried powder at −20 °C and was rapidly utilized in further experiments to avoid the sample aging. The crude protein (N × 6.25, as determined by the Kjeldahl method), ash and moisture contents of NSI sample were 90.25%, 3.08% and 2.10% w/w, respectively. The storage globulins 7S and 11S are in native state, as was corroborated by differential scanning calorimetry (DSC, Q200 calorimeter, TA Instruments, DE, USA): the DSC thermogram showed two endothermic peaks with a total denaturation enthalpy of ~17 J/g, according to previous paper (Sorgentini & Wagner, 1999).

2.2.2. Preparation of aqueous dispersions

NSI aqueous dispersions (0.5, 1.0 and 2.0% protein w/v) were prepared by dissolving the freeze-dried sample in 10 mM sodium phosphate buffer pH 7.0; then were stirred with a magnetic stirrer for at least 2 h to ensure complete dispersion of sample prior to preparation of emulsions. Stirring was gentle to minimize foam formation. Sodium azide (0.02% w/v) was added in order to retard the microbial growth. SC aqueous dispersions were prepared by following the same experimental procedure described above. Total denaturation of NSI was achieved by heating the corresponding aqueous dispersions (0.5, 1.0 and 2.0% w/v) at 90 °C for 5 min and further cooling in a water-ice bath to room temperature. Total denaturation of 7S and 11S proteins in this sample was also confirmed by DSC thermograms, which showed no endothermic peaks. This condition of thermal treatment ensures the total denaturation of storage globulins with a minimal impact on protein solubility (Mitidieri & Wagner, 2002). The denatured soy isolate (DSI) aqueous dispersions were immediately utilized after their preparation.

2.2.3. Determination of protein solubility

Protein solubility of NSI, DSI and SC aqueous dispersion (0.5, 1.0 and 2.0% w/v in 10 mM sodium phosphate buffer pH 7.0) was determined by following the experimental procedure of Mitidieri and Wagner (2002). Dispersions were centrifuged for 30 min at 15,000*g* (Beckman Coulter GS-15R high-speed centrifuge, F2402 rotor, Beckman Coulter Inc., Fullerton, USA), and the protein concentration of the supernatants was determined by the method of Markwell, Haas, Bieber, and Tolbert (1978), using serum albumin as standard. This method agreed well with the Kjeldahl method (data not shown). Protein solubility (PS %) was calculated using the following formula:

$$\text{PS}\ (\%) = (\text{protein content in supernatant}/\text{initial protein content}) \times 100 \quad (1)$$

Assays were conducted at least in triplicate.

2.2.4. Preparation of o/w emulsions

Oil-in-water emulsions were prepared by mixing refined sunflower oil with aqueous protein dispersions (0.5, 1.0 and 2.0% w/v of NSI, DSI or SC in 10 mM sodium phosphate buffer pH 7.0), in a high-speed blender (Ultraturrax T-25, S25-20NK-19G dispersing tool, IKA Labortechnik, GmbH & Co, Staufen, Germany) at 20,000 rpm for 30 s. Pre-emulsions (oil volume fraction, $\phi = 0.25$) were then re-circulated through a twin-stage valve high pressure homogenizer (Panda 2K, GEA Niro Soavi, Parma, Italy) for 6 min, sufficient to achieve four passes through the valves. The homogenization pressure was 40 MPa and 10% of total pressure was maintained over the second valve.

2.2.5. Freeze-thaw protocol

Freeze-thaw treatments of o/w emulsions and aqueous dispersions were performed immediately their preparation. Samples (20 ml) were transferred to vertical plastic containers (internal diameter = 30 mm) and were isothermally stored in still air for 24 h at −20 °C. The temperature of samples before freezing experiments was set at 20 ± 1 °C. After storage, frozen samples were thawed into a water bath at 20 ± 1 °C, and kept at this temperature before further characterization analyses. After freeze-thaw, some of the emulsions separated into a number of layers with different visual appearances. The thickness of each of the layers formed was determined visually using a ruler. The stability of emulsions to gravitational separation (GS), represented by the ratio in percentage of bottom serum height (h_S) respect to total sample height (h_T) was calculated as:

$$GS\ (\%) = (h_S/h_T) \times 100 \quad (2)$$

2.2.6. Particle size distribution

The particle size distribution (PSD) of initial and freeze-thawed emulsions was determined as differential volume in the diameter range of 0.1–1000 μm by laser scattering using a Malvern Mastersizer 2000E (Malvern Instruments Ltd, Worcestershire, UK). Optical parameters applied were: refractive indexes of sunflower oil and water 1.47 and 1.33, respectively; adsorption: 0.001. Before particle size measurements, emulsions were carefully mixed by turning the containers upside down to get a droplet size for the whole sample. Then, two aliquots of each emulsion were diluted separately with 10 mM sodium phosphate buffer pH 7.0 without and with 1.0% w/v sodium dodecyl sulphate (SDS). Usually, original droplet aggregates are eliminated if SDS is present during the diffraction analysis, due to this molecule is able to displace proteins from oil/water interface and to induce electrostatic repulsions between droplets due to its negative charge (Smulders, Caessens, & Walstra, 1999). Therefore, SDS decreases the extent of bridged oil droplets, but it would not breakdown aggregates if the droplets were coalesced. In measuring without SDS, dilution and stirring were likely to disrupt any weakly flocculated droplets, but leave strongly flocculated droplets intact; therefore this method allows evaluating flocs stable in the measurement conditions, which are formed by a bridging mechanism (Thanasukarn et al., 2004). In addition, the laser light scattering pattern obtained by each sample is processed by using the Mie theory, which describes spherical, isotropic and homogeneous particles. Consequently, the particle size distributions of flocculated emulsions should be analyzed with caution because flocs could not match these characteristics, and data reported relate to the equivalent Mie sphere (Castellani, Belhomme, David-Briand, Guérin-Dubiard, & Anton, 2008). Notwithstanding this, a large increase in $D_{4,3}$ reflects the association of individual droplets into larger flocs (Anton, Beaumal, Brossard, Llamas, & LeDenmat, 2002).

The samples prepared with and without SDS, were dispersed in 600 ml of water at 2000 rpm in the dispersion unit (Hydro 2000 MU, Malvern Instruments Ltd, Worcestershire, UK). From particle size distributions, the Sauter mean (surface-weighted, $D_{3,2}$) and De Brouckere (volume-weighted, $D_{4,3}$) moment mean diameters were obtained, both expressed in micrometers.

2.2.7. Adsorbed protein percentage

Adsorbed protein percentage (AP %) of emulsions was estimated by following the experimental procedure reported by Mitidieri and Wagner (2002) with slight modifications. Emulsions were centrifuged at 18,000*g* for 30 min to accelerate the creaming (Beckman Coulter GS-15R high-speed centrifuge, F2402 rotor, Beckman Coulter Inc., Fullerton, USA). The lower aqueous phase was carefully withdrawn with a hypodermic syringe and the centrifugation was repeated to assure the total separation of smallest droplets. AP % was determined as:

$$AP\ \% = [(C_{initial} - C_{aq})/C_0] \times 100 \quad (3)$$

$C_{initial}$ and C_{aq} are the protein concentration of initial aqueous dispersion and lower aqueous phase after centrifugation of emulsions, respectively; C_0 is the protein concentration of supernatant obtained from centrifugation of aqueous dispersion in the same conditions (18,000*g*, 30 min) (Mitidieri & Wagner, 2002). The protein content was determined by the method of Markwell et al. (1978), using bovine serum albumin as standard.

Measurements were performed at least twice.

2.2.8. Flocculation

Flocculation degree (FD %) was calculated both in initial and freeze-thawed emulsions, employing the size particle data, as:

$$FD\ \% = [(D_{4,3} - D_{4,3\ SDS})/D_{4,3\ SDS}] \times 100 \quad (4)$$

$D_{4,3}$ and $D_{4,3\ SDS}$ are the volume-weighted diameters, measured in the absence and presence of SDS, respectively (Palazolo et al., 2005). This parameter only describes the tendency to droplet flocculation, regardless the droplet coalescence.

2.2.9. Coalescence degree

The effect of freeze-thawing on emulsion stability was evaluated through the coalescence destabilization and measurement of the amount of free oil (oiling off). Coalescence degree (CD %) was calculated from:

$$CD\ (\%) = \left[\left(D_{4,3\ SDS\ f\text{-}t} - D_{4,3\ SDS\ in}\right)\Big/ D_{4,3\ SDS\ in}\right] \times 100 \quad (5)$$

$D_{4,3\ SDS\ f\text{-}t}$ and $D_{4,3\ SDS\ in}$ are the volume-weighted mean diameters of freeze-thawed and initial, non-frozen, emulsions, respectively. Both parameters were obtained from particle size distribution measurements in the presence of 1% w/v of SDS.

2.2.10. Oiling off

The dye-dilution technique was utilized to determine the oiling off of o/w emulsions after freeze-thawing. The experimental procedure of Palanuwech, Poniteni, Roberts, and Coupland (2003) was followed with some modifications. Sudan III (Solvent Red 23) solutions (0.0015 w/w %) were prepared by dissolving the adequate quantity of liposoluble dye in refined sunflower oil under gently magnetic stirring overnight. The absorbance spectrum of Sudan III in sunflower oil exhibited a single absorbance maximum of 508 nm. Therefore, quantitative readings at this wavelength were made in a PG T-60 UV–visible spectrophotometer (PG Instruments, Leicester, UK), using polystyrene cuvettes and refined sunflower oil as blank. In order to determinate the amount of free oil, 4 g of dye solution was gently mixed with 16 g of emulsion sample, incubated for 60 min and then centrifuged at 700*g* for 20 min (Rolco CM 4080 Millenium low-speed centrifuge, Buenos Aires, Argentina). An aliquot of dyed free oil on top of emulsion (1.5 ml) was carefully sucked off with a Pasteur pipette and centrifuged at 15,000*g* for 20 min (Beckman Coulter GS-15R high-speed centrifuge, F2402 rotor, Beckman Coulter Inc., Fullerton, USA). The absorbance of supernatant at 508 nm was then determined. The change in the absorbance due to dye dilution was related to the oiling off, according to the following expression:

$$\text{Oiling off}\ (\%) = [M_o \times (A - 1)/(M_e \times \phi_e)] \times 100 \quad (6)$$

M_o is the mass (in g) of added dye solution, M_e is the mass (in g) of emulsion, ϕ_e is the mass fraction of oil in the emulsion and $A = A_b/A_a$ is the ratio of the measured absorbances of the dye before (A_b)

and after (A_a) extraction process. The corresponding procedures of calibration were carried out according to previous works (Palanuwech et al., 2003). The oiling off in the emulsion samples (w/w %) was determined from a calibration curve prepared using emulsions with known amount of free oil on top.

2.2.11. Optical microscopy

Optical microscopy was used to visualize the microstructure of initial, non-frozen emulsions. Observations were made from emulsions, which were diluted 10-fold in 10 mM sodium phosphate buffer pH 7.0 at room temperature. An optical microscope (with adapted USB MC-280 video camera), using differential interference contrast mode at 400× magnification, was employed.

2.2.12. Differential scanning calorimetry

The thermal behavior of o/w emulsions was determined by differential scanning calorimetry (DSC, Q200 calorimeter, TA Instruments, DE, USA). Emulsions were frozen according to experimental procedure mentioned above (Section 2.2.5). Then, aliquots of frozen emulsions (10–15 mg) were placed in aluminum pans and rapidly transferred to calorimeter alongside an empty reference pan. The calorimeter oven was previously cooled to −20 °C. Then, samples were isothermically kept at this temperature for 15 min and then heated to 20 °C at 1 °C min^{-1}. A unique endothermic transition attributed to ice melting was observed. The enthalpy was measured for a known mass of emulsion and for a known mass of pure water. The amount of freezable water (FW %) in emulsions was calculated as FW % = $(\Delta H_E/\Delta H_W) \times 100$ where ΔH_E and ΔH_W were the enthalpy changes per unit of mass of water for emulsions and pure water respectively. In this DSC assay, the cooling was not performed inside the pan due to this condition do not reproduce the freezing treatment of o/w emulsion at bulk scale. Large degree of supercooling both in the water and oil phase is common in small, static samples.

Assays were conducted at least in triplicate.

2.2.13. Transmission electron microscopy

Transmission electron microscopy (TEM) was carried out on the emulsions before and after freeze-thaw treatment. Briefly, samples without previous dilution were collected on copper grids (Formvar/carb, 300 mesh) and subsequently stained using saturated uranyl acetate solution for 1 min. The electron microscope (Phillips EM-301, Netherlands) was operated at 60 kV. A magnification of 46,000× was employed. Micrographs were taken from different areas of the sample grid and three or four images were acquired using digital image capture system.

2.2.14. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and differences between mean values by the Fisher's Least Significant Differences (LSD). Statistical analysis was carried out using Statgraphics Centurion XV software (StatPoint Inc. 2005, USA). Significance was considered at $p < 0.05$.

3. Results and discussion

3.1. Characterization of initial emulsions

Freezing treatment of o/w emulsions were carried out immediately after their preparation. At this time, NSI, DSI and SC emulsions were completely stable to gravitational separation, independently of protein concentration. The particle size, adsorbed protein percentage and flocculation degree of unfrozen emulsions are shown in Table 1. As protein concentration increases, a sharp decrease in Sauter mean diameter ($D_{3,2}$) was effectively observed in soy protein-stabilized emulsions, both in the absence and presence of SDS. In contrast, SC emulsions at 1.0 and 2.0% w/v exhibited similar $D_{3,2}$ values and only a slight increase of particle diameter was evidenced at low protein concentration (0.5% w/v). At 2.0% w/v, no significant differences were observed in $D_{3,2}$ parameter between NSI, DSI and SC emulsions ($p < 0.05$). In addition, percentages of adsorbed protein (AP %) was high for all emulsions (>60). For three emulsions, AP % decreased with the increase of protein concentration. Thermal denaturation of soybean proteins caused an increase of AP % of resultant emulsions in whole range of protein concentration (Table 1).

Table 1
Sauter mean diameter ($D_{3,2}$), adsorbed protein (AP %), and flocculation degree (FD %) of o/w emulsions ($\phi = 0.25$) prepared with native soybean isolate (NSI), thermally-denatured soybean isolate (DSI) and sodium caseinate (SC) at different protein concentrations (0.5, 1.0 and 2.0% w/v).

Emulsion	Sample concentration (w/v)	$D_{3,2}$ (μm)		Adsorbed protein (AP %)	FD (%)
		−SDS	+SDS		
NSI	0.5	4.98 ± 0.82	1.55 ± 0.19	80.6 ± 3.0	1316.8 ± 131.0
	1.0	0.87 ± 0.14	0.69 ± 0.10	74.3 ± 1.3	183.1 ± 18.7
	2.0	0.45 ± 0.01	0.44 ± 0.01	64.8 ± 2.4	18.2 ± 5.3
DSI	0.5	4.92 ± 0.07	1.31 ± 0.01	88.1 ± 2.6	396.4 ± 31.5
	1.0	0.98 ± 0.19	0.73 ± 0.03	82.6 ± 3.3	80.4 ± 25.4
	2.0	0.49 ± 0.01	0.46 ± 0.01	72.0 ± 3.2	35.1 ± 2.1
SC	0.5	0.51 ± 0.02	0.53 ± 0.02	80.3 ± 2.0	0
	1.0	0.43 ± 0.01	0.44 ± 0.01	79.0 ± 2.3	0
	2.0	0.41 ± 0.02	0.42 ± 0.01	71.3 ± 5.0	0

Volume-weighted diameters ($D_{4,3}$) are more sensitive to droplet aggregation processes than Sauter mean diameters ($D_{3,2}$) (Cortés-Muñoz, Chevalier-Lucia, & Dumay, 2009; Relkin & Sourdet, 2005). Therefore, the particle size distribution (PSD) of fresh and freeze-thawed emulsions was expressed as volume frequency and $D_{4,3}$ values were used in order to evaluate the destabilization degree (equations (4) and (5)). Fig. 1 shows comparatively the PSD for NSI and DSI emulsions. At 0.5 and 1.0%, NSI emulsions exhibited bimodal particle size distributions, whereas they were monomodal when the measurements were carried out with SDS (Fig. 1). In the bimodal distributions, the peaks at higher particle size were the result of the presence of flocs formed by a bridging mechanism. For 0.5% NSI emulsions, most of volume of emulsified oil was distributed in large particles (10–100 μm) (Fig. 1a). As protein concentration increases (1.0% w/v), a shift of both peaks towards smaller diameters was detected: this emulsion also exhibited a narrower distribution, with a particle population in the range 0.1–10.0 μm (Fig. 1b). At 2.0% w/v, the PSD for NSI emulsion was monomodal: a peak in the range 0.1–4.0 μm was effectively observed (Fig. 1c), indicating that increasing protein concentration results in a less flocculated emulsion. In the presence of SDS, the peaks in monomodal distributions were also displaced towards smaller particle diameters when protein concentration increases, suggesting a decrease in size of individual droplets (Fig. 1d–f): $D_{3,2}$ clearly decreased in the sequence of protein concentrations: 0.5% > 1.0% > 2.0% (Table 1).

At 0.5% w/v, DSI emulsions exhibited a smaller particle size respect to those prepared with NSI. This effect was observed both in absence and in presence of SDS (Fig. 1a, d). A main particle population was observed in the range 2–20 μm plus a secondary population close to 1 μm (Fig. 1a). At higher protein concentrations, PSD of NSI and DSI emulsions were similar (Fig. 1b, c). When the PSD were measured in the presence of SDS, a monomodal character was always observed, independently of protein concentration. Simultaneously, a shift of population towards smaller particle sizes was also detected as protein concentration increases (0.5–2.0% w/v) (Fig. 1d–f); these results suggest again a decrease in the size of individual droplets.

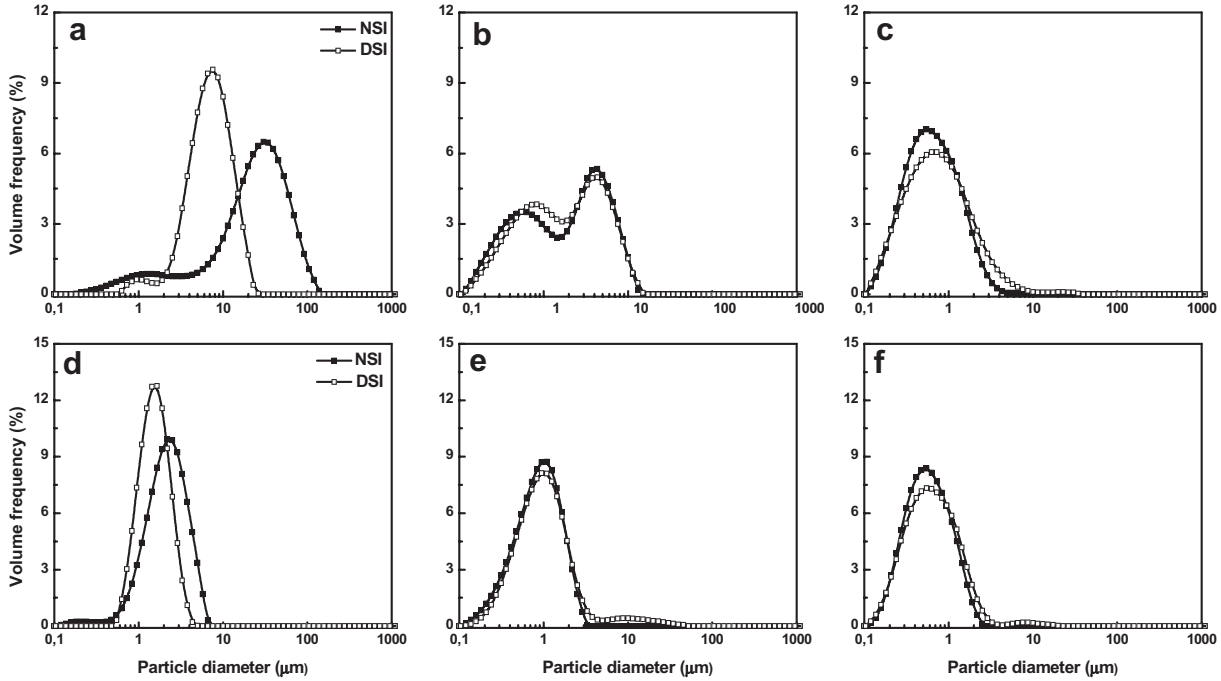

Fig. 1. Particle size distributions (PSD, expressed as volume frequency) of initial, unfrozen o/w emulsions prepared with aqueous dispersions of native and thermally-denatured soybean isolates (NSI and DSI, respectively) at different protein concentrations. a, d: 0.5%; b, e: 1.0%; c, f: 2.0% w/v. Measurements were performed in the absence (a, b, c) and presence (d, e, f) of 1.0% w/v SDS.

All emulsions prepared with SC exhibited a monomodal PSD, both in the absence and presence of SDS (data not shown), so no bridged flocs exist to be broken by SDS. This result was similar to those reported by Carrera Sánchez and Rodríguez Patiño (2005) in a previous paper. The emulsifying behavior of SC was higher than soy protein, which was more evident in emulsions prepared with more dilute dispersions. $D_{3,2}$ values of 0.5% w/w SC were significantly lower than those of NSI and DSI emulsions ($p < 0.05$) (Table 1).

From volume-weighted diameter values ($D_{4,3}$), obtained from PSD measured without and with SDS, flocculation degree (FD %) of fresh emulsions was obtained (Table 1). NSI showed the highest FD % at low protein concentration (0.5% w/v). Moreover, for NSI and DSI emulsions, this parameter exhibited a noticeable decrease with the increase of protein concentration. It can be clearly observed that the thermal denaturation of soybean proteins produced a decrease of FD %. Unlike SC emulsions, which were not flocculated in the whole range of concentration, in NSI and DSI emulsions bridging flocculation was almost inhibited only at 2% w/v. In addition, optical microscopy was used to provide additional information on microstructure of initial, non-frozen emulsions. Micrographs of o/w emulsions, diluted 10-fold in 10 mM sodium phosphate buffer, are showed in Fig. 2. For 0.5 and 1.0% NSI and DSI emulsions, the presence of flocs was effectively observed. This result is in accordance with those obtained from PSD (Fig. 1, Table 1).

According to McClements (2004) protein emulsifiers differs in the rate at which they absorb to droplet surface during homogenization, at the minimum amount that is required to saturate the droplet surface and their ability to protect droplets against coalescence under different environmental conditions. NSI is constituted by storage proteins, 11S and 7S, which are globular, with a high molecular size and complex quaternary structure. The interfacial behavior studies carried out by Tornberg et al. (1997) suggested that slow conformational changes at the oil interfaces occur with soy proteins, due to their structural characteristics. In contrast, a quick conformational change on adsorption in SC proteins has been reported by these authors. In addition, when the emulsifier concentration is limiting, i.e. there is not enough sufficient protein to cover the interface, the particle size is governed primarily by the emulsifier concentration rather the energy input of the homogenizer (McClements, 2004). Based on these observations, at low protein concentration (0.5–1.0% w/v) is expected that NSI emulsions exhibits higher FD % and $D_{3,2}$ values respect to those prepared with SC. In contrast, at 2.0% w/v there is more protein present than is required to completely cover to droplet surface created by the homogenizer. This fact explain the similar values of $D_{3,2}$ for NSI and SC emulsions at high protein concentration (Table 1).

Total denaturation of soy globulins (7S and 11S) caused by thermal treatment at pH 7.0 of dilute aqueous dispersions led to unfolding of molecules, exposing hydrophobic zones (Yamauchi et al., 1991). The thermal treatment in these conditions also induces partial dissociation of 11S globulin and formation of soluble aggregates, corroborated by SDS-PAGE and FPLC studies (Palazolo et al., 2004; Sorgentini et al., 2002). A significant increase of aromatic surface hydrophobicity (around 3 times, determined by 1-anilino 8-napthalene sulphonate fluorescent probe) as a consequence of thermal denaturation was reported in previous papers (Mitidieri & Wagner, 2002; Palazolo et al., 2003; Sorgentini et al., 2002). These physicochemical modifications on soy globulins are able to provide a better behavior for the adsorption at the oil-water interface, especially at low protein concentration, where a significant decrease of FD % of DSI emulsions was observed (Table 1). Nonetheless, as in the emulsions prepared with NSI, at 0.5 and 1.0% w/v the protein concentration is limiting during homogenization process and bridging flocculation also occur. As was previously reported (Keerati-u-rai & Corredig, 2009; Puppo et al., 2005), soluble aggregates of denatured soy isolates can be adsorbed at the oil-water interface during homogenization, which are in accordance with the increase of AP % of DSI emulsions respect to those prepared with NSI in whole range of protein concentration (Table 1).

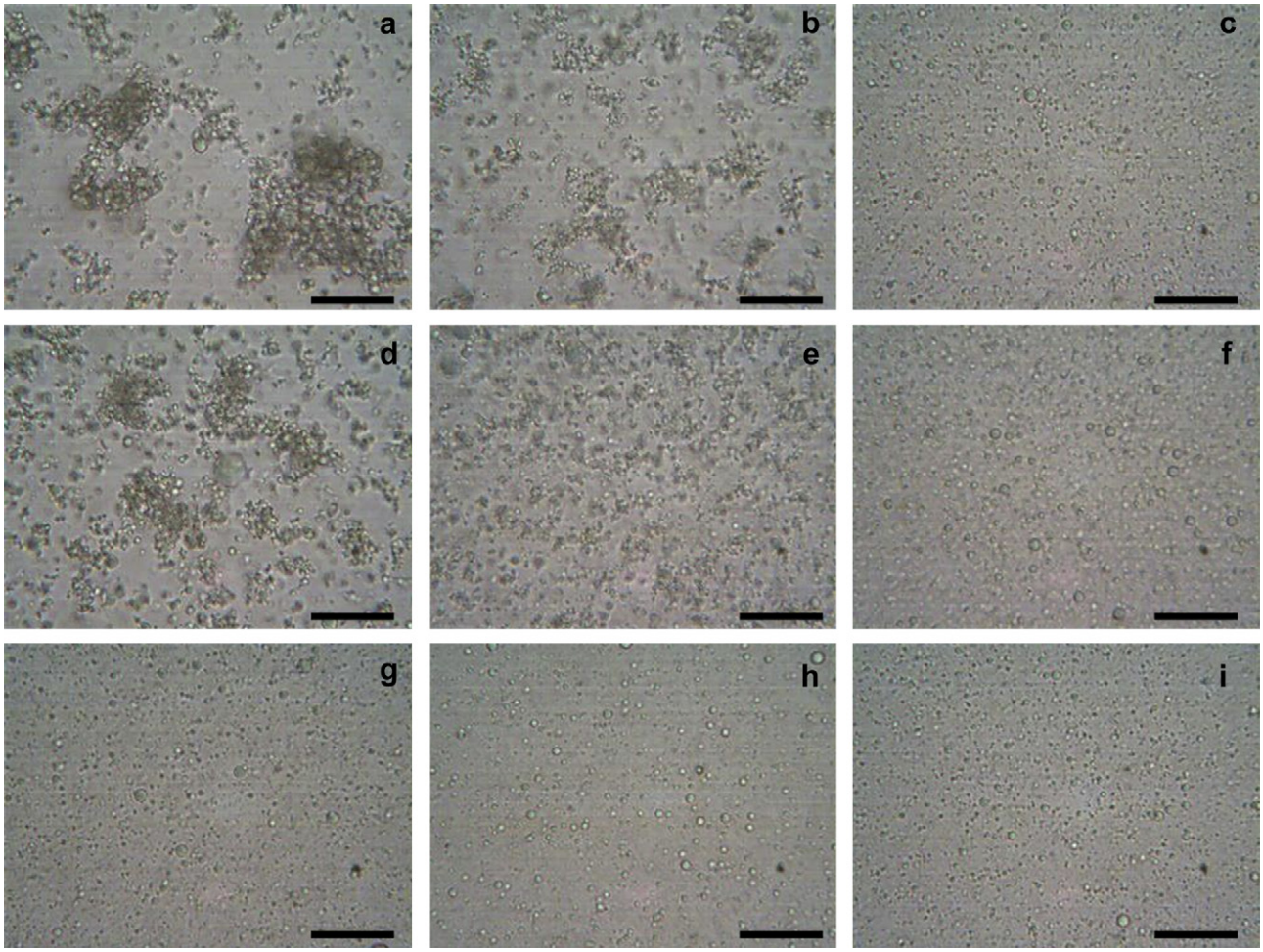

Fig. 2. Optical micrographs of initial, unfrozen, o/w emulsions prepared with different protein samples. a, b, c: NSI 0.5, 1.0 and 2.0% w/v; d, e, f: DSI 0.5, 1.0 and 2.0% w/v; g, h, f: SC 0.5, 1.0 and 2.0% w/v. Emulsions were diluted 10-fold in the same buffer used to prepare them. Scale bar is 10 μm.

Finally, the obtained results showed noticeable differences between NSI, DSI and SC emulsions, even though they were prepared with aqueous dispersions of similar concentration, at the same conditions of homogenization. These differences in particle size, flocculation degree, and adsorbed protein percentage were more pronounced in o/w emulsion prepared at low protein concentration.

3.2. *Effect of freeze-thaw treatment on stability of o/w emulsions*

When o/w emulsions are cooled to temperatures where only the lipid phase becomes partially crystalline, the protein-stabilized emulsions are generally stable. This fact has been attributed to the ability of proteins to form relatively thick interfacial films that are difficult to penetrate to fat crystals. However, when these emulsions are cooled at subzero temperature, the interfacial film become more prone to disruption due to additional destabilizing effect of the crystallization of water in the continuous phase (McClements, 2004). In this work, sunflower oil was utilized as dispersed phase. DSC assay showed that the crystallization of bulk oil starts at −17 °C, in agreement with other works (Roos, 1995). In bulk liquids, impurities can act as sites for nucleation and crystallization can proceed rapidly by a heterogeneous mechanism. However, as was mentioned above, when the liquid is finely divided into emulsion droplets so that the number of oil droplets is much larger than the number of impurities, the crystallization must arise spontaneously by a homogeneous nucleation mechanism (Cramp et al., 2004). This explains the large degree of undercooling of emulsified oil phase in o/w emulsions (McClements, 2004). In our work, o/w emulsions were subjected to freezing at −20 °C (only 3 °C below the onset of crystallization of sunflower oil) for 24 h, so that it is probable that the lipid phase remains almost completely in liquid state. This assumption was corroborated by DSC assays as described in Materials and Methods (Section 2.2.12), where the heating curve of frozen emulsions exhibited only an endothermic peak (onset temperature = −1 °C) corresponding to melting to aqueous phase. Based on these assays, we can remark that oil do not crystallizes in emulsions droplets during the frozen storage at −20 °C for 24 h. Hence, the destabilization phenomena induced by freeze-thaw in o/w emulsions would be mainly attributed to destabilizing effect of ice formation during frozen storage.

The amount of freezable water (FW %) was higher than 97% in all emulsions, whatever the sample type and concentration ($p < 0.05$). Nonetheless, after freeze-thaw treatment, we observed a noticeable difference between stability of soy protein and SC-stabilized emulsions. Photographs of thawed emulsions prepared with these protein samples are shown in Fig. 3. At low protein concentration (0.5 w/v), SC samples separated into two layers (a droplet-rich creamy layer and droplet-depleted turbid layer). Most of dispersed phase of SC emulsions remained in an emulsified state whatever the protein concentration: the amount of free oil was <2% (Table 2). In the opposite, NSI and DSI emulsions were broken by the freeze-

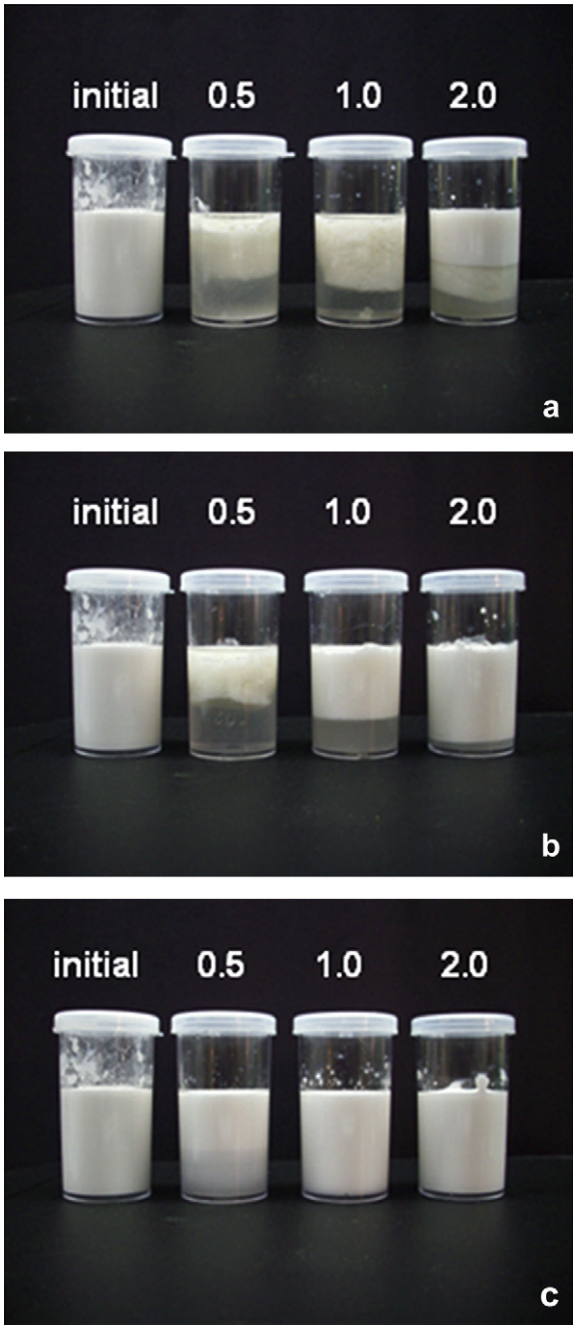

Fig. 3. Photographs of freeze-thawed o/w emulsions prepared with (a) NSI (b) DSI and (c) SC at different protein concentrations (0.5, 1.0 and 2.0% w/v). Emulsions were frozen at −20 °C and kept in the frozen state at the same temperature for 24 h. Frozen emulsions were thawed by immersion in a water bath at 20 °C. For comparative purposes, initial, unfrozen, emulsions for each protein sample (2% w/v) were also included.

thawing process, separating into three layers: a top layer of free oil, an intermediate coagulated cream layer and an aqueous turbid layer in the bottom. At 0.5% w/v, the oiling off of soybean protein emulsions was >25% (Table 2), which is consistent with their visual appearances (Fig. 3 a, b). When emulsions were prepared with aqueous dispersions of higher protein concentration (2.0% w/v), photographs at bulk scale showed an enhancement of freeze-thaw stability: SC emulsions were apparently stable and those prepared with soybean proteins exhibited gravitational separation. For soy protein-stabilized emulsions, the lowest values of gravitational separation (GS %) were observed with those prepared with more

Table 2
Destabilization parameters of o/w emulsions ($\phi = 0.25$) of NSI, DSI and SC emulsions after freeze-thawing.

Sample	Concentration (w/v)	CD (%)	FD (%)	Oiling off (%)	GS (%)
NSI	0.5	10,462 ± 175	55 ± 5	28.9 ± 1.7	35 ± 2
	1.0	23,643 ± 1200	38 ± 4	6.0 ± 0.1	33 ± 0
	2.0	13,040 ± 88	45 ± 6	1.7 ± 0.3	17 ± 2
DSI	0.5	10,656 ± 60	41 ± 4	26.9 ± 2.3	56 ± 2
	1.0	331 ± 11	2001 ± 25	4.8 ± 0.3	26 ± 2
	2.0	86 ± 1	3358 ± 31	1.6 ± 0.4	4 ± 1
SC	0.5	99 ± 7	201 ± 6	1.9 ± 0.4	38 ± 2
	1.0	25 ± 1	6	1.5 ± 0.4	0
	2.0	11 ± 1	0	1.0 ± 0.3	0

concentrated solutions DSI emulsion exhibited a low value of GS % (4%), while the NSI emulsion gave a GS value of 17%. Furthermore, at 2.0% (w/v) free oil layer was not observed in NSI and DSI emulsions (Fig. 3a, b), which was in accordance with negligible values of oiling off (<2%, Table 1).

Fig. 4 shows comparatively the PSD of NSI, DSI and SC emulsions after freeze-thawing. Both, in the absence and presence of SDS, it can be observed that the higher the concentration of sample, particle populations are smaller. PSD in the absence of SDS showed that NSI emulsions at 0.5 and 1.0% exhibit a major population at >100 μm, but at 2.0% a bimodal character was observed, with two particle populations in a wide range of particle diameters (2–700 μm). When the PSD measurement was performed with 1.0% w/v SDS, a same PSD was obtained for NSI emulsions at 0.5 and 1.0% w/v. On the contrary, for 2.0% NSI emulsions a bimodal PSD with lower particle size was observed with SDS. The peak at 10 μm in PSD without SDS disappeared totally in the presence of deflocculating agent, conducting to a new population at particle size < 2 μm (Fig. 4). This result is attributed to the presence of flocs induced by freeze-thaw process. In addition, a peak at higher particle size (>70 μm) was observed both in the absence and presence of SDS, which is consistent with a coalescence destabilization induced by freeze-thawing. At low protein concentration (0.5% w/v), freeze-thawed DSI emulsions did not resist the stress induced by freeze-thawing and hence, were destabilized by coalescence. When PSD measurements were preformed without and with SDS, a particle population at sizes higher than 70 μm was always observed. At 1.0% w/v a trimodal PSD were obtained in the absence of SDS, with two main peaks in the ranges 2–30 and 30–1000 μm plus a smaller peak in the submicron range. The presence of these particle populations could be attributed mainly to droplet aggregates that SDS was able to dissociate into particles < 3 μm; moreover, in the presence of surfactant, the minor peak in the range 20–100 μm for DSI emulsion is consistent to droplet coalescence. When the sample concentration increased from 1.0 to 2.0% w/v, a trimodal PSD was also observed. However, the peaks at high particle size (2–20 μm and 20–300 μm) were consistent with the presence of flocs, due to they disappeared when PSD measurements were carried out with SDS (Fig. 4). For SC emulsions under freeze-thawing, monomodal PSD were observed for all the concentrations tested. At 0.5%, the PSD shows a population with a maximum at 7 μm and 2 μm, when measured with and without SDS, respectively, indicating the presence of flocs. At 1.0–2.0%, SC emulsions are totally stable: PSD was similar (maximum at 0.8–1 μm) both without and with SDS (Fig. 4).

From the $D_{4,3}$ values of initial and freeze-thawed emulsions, flocculation degree (FD%, equation (4)) and coalescence degree (CD %, equation (5)), were calculated for emulsions prepared with different protein samples (Table 2). For NSI emulsions high CD % values were obtained ($>10^4$), suggesting a high degree of instability.

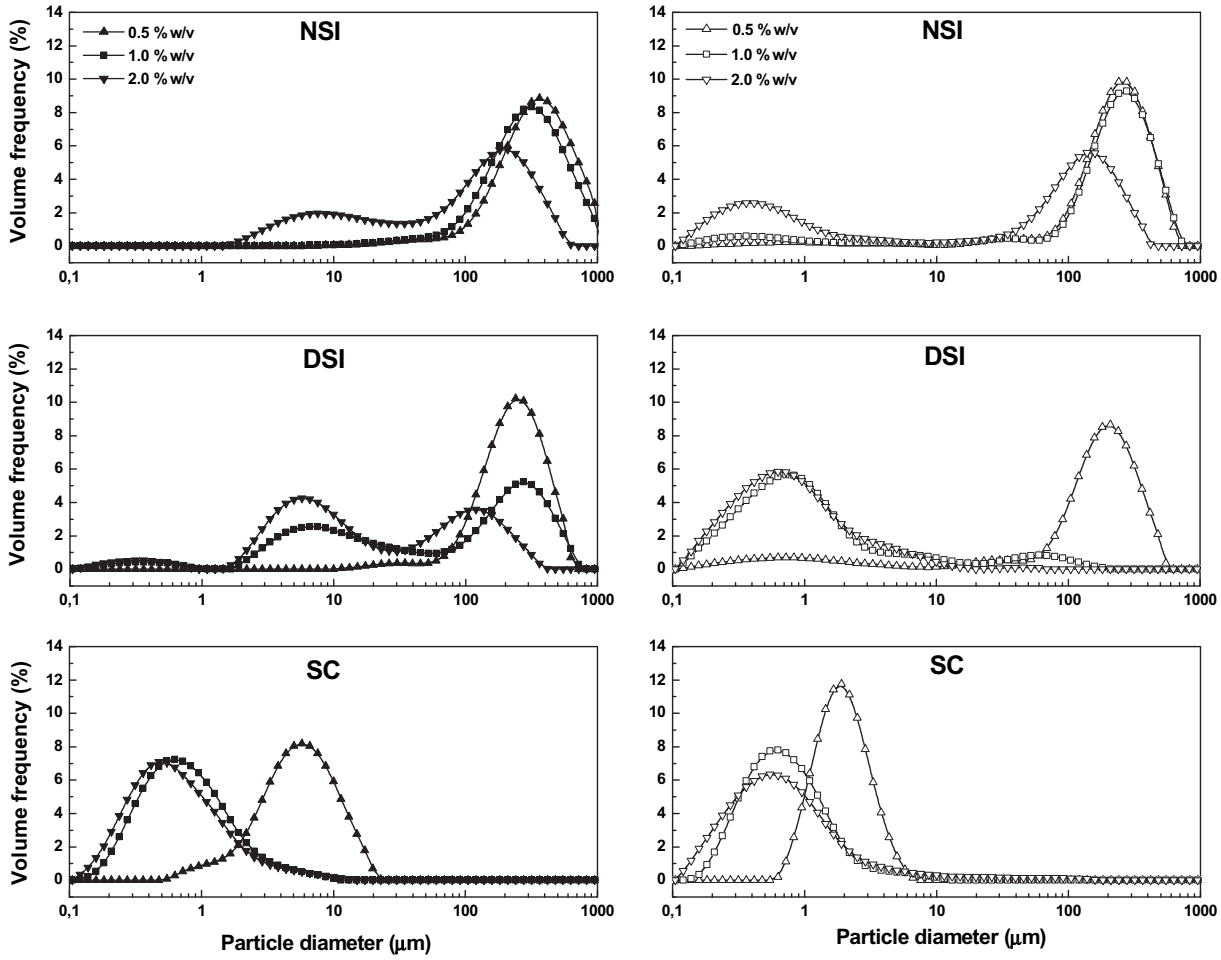

Fig. 4. Particle size distributions (PSD, expressed as volume frequency) of freeze-thawed o/w emulsions prepared with NSI, DSI and SC samples at 0.5, 1.0 at 2.0% w/v, in the absence (filled symbols) and presence (open symbols) of 1.0% w/v of SDS. Emulsions were frozen at −20 °C and kept in the frozen state at the same temperature for 24 h. Before PSD measurements, frozen emulsions were thawed in a water bath at 20 °C.

CD % was the highest for emulsion prepared with 1.0% NSI. However, this result is associated with a lesser value of initial $D_{4,3}$ for this emulsion (0.96 ± 0.03 μm) respect to those prepared with the more diluted aqueous dispersion (2.16 ± 0.01 μm). For 0.5% w/v NSI emulsions, the formation of visible oil at the upper part of container (oiling off $= 28.9 \pm 1.7\%$) was the result of extensive coalescence process, whereas the amount of free oil was relatively low in 1.0% NSI emulsion (Fig. 3a, Table 2). Therefore, in order to evaluate the coalescence stability, CD % and oiling off (%) values must always be analyzed together. Furthermore, the FD % values of thawed emulsions prepared with NSI (0.5 and 1.0% w/v) were significantly lesser than in non-frozen emulsions (Tables 1 and 2), suggesting that the flocs are destabilized by coalescence. For 2.0% NSI, the high value of CD % is in accordance with the PSD measured in the presence of SDS, where a peak at particle diameters > 60 μm was observed (Fig. 4). Moreover, in this latter case, FD % was slightly higher than in initial emulsion due to presence of droplet aggregates that resist the stress induced by freeze-thaw process. The gravitational separation observed in NSI emulsion is the result of the presence of flocs and coalesced droplets. On the other hand, for DSI emulsions the increase of protein concentration dramatically improved their stability to coalescence. Simultaneously, the FD % values showed a marked increase respect to initial values for 1.0 and 2.0% DSI emulsions (25 and 135 times, respectively), suggesting the formation of flocs stable to freeze-thawing (Tables 1 and 2).

As was mentioned above, we observed a noticeable difference between freeze-thaw stability of soybean and SC-stabilized emulsion (Fig. 3). Only the emulsion prepared with 0.5% SC exhibited gravitational separation, due to formation of coalesced droplets (CD % ∼ 100) and flocs (FD % ∼ 200) during freeze-thawing. The increase of protein concentration (0.5–2.0% w/v) enhanced the stability to coalescence and flocculation (Table 2). These results are consistent with the visual appearances of thawed emulsions, as indicated in Fig. 3c. According to results mentioned above, a comparative analysis indicates that the coalescence stability was decreased in the sequence SC > DSI > NSI whatever the protein concentration.

Several destabilizing effects can occur in o/w emulsions in frozen state, but the most important belong to two groups. First, the crystallization of water molecules leads to the formation of a freeze-concentrated unfrozen aqueous phase, which is concentrated in all aqueous solutes. Then, the ionic strength of the aqueous phase increases and pH decreases. High concentration of salts can screen out any electrostatic repulsion between droplets and make easier to force into close proximity (Ghosh et al., 2006; McClements, 2004). Second, the dispersed phase volume fraction increases as the amount of liquid water is reduced during the freezing process. The increase of oil volume fraction (or oil mass fraction) decreases the stability of emulsions to freeze-thawing (Cortés-Muñoz et al., 2009; Ghosh et al., 2006). When ice crystals form in the aqueous phase, the oil droplets are forced close together. The actual volume fraction in

frozen state will approach close packing for typical emulsion. The aggregation processes, such as flocculation and coalescence are favored when the droplets are forced into close proximity (Ghosh & Coupland, 2008; McClements, 2004; Thanasukarn et al., 2004).

As mentioned above, the emulsions prepared with 1.0 and 2.0% of SC were almost totally stable to coalescence and flocculation processes during freeze-thaw treatment. The similar values of $D_{3,2}$ for unfrozen emulsions would indicate that the amount of protein is sufficient to cover the interface (Table 1). It seems that these results are not in agreement with those previously obtained by other authors. Thanasukarn et al. (2004) reported a fully destabilization of emulsions prepared with sodium caseinate and hydrogenated palm oil. A high degree of fat destabilization was observed for SC emulsions both in differential scanning calorimetry and in isothermal storage experiments. Ghosh et al. (2006) reported a high degree of destabilization of hexadecane oil-in-water emulsions ($\phi_m = 0.40$) prepared with 2% w/w sodium caseinate. The discrepancy of our results respect to those mentioned above can be explained by many reasons. Hydrogenated palm oil and hexadecane crystallize during the frozen storage. When emulsions are subjected to freeze-thawing, they will be stable only if the interfacial film remains intact. One role of type of lipid is to affect the ways the forces produced by the expanding ice is transmitted to the membrane. According to Ghosh & Coupland (2008), under the pressure of ice, a crystalline droplet is presumably unable to deform and hence is less resistant to rupture. Moreover, a concentrated emulsion ($\phi_m = 0.40$) is more prone to destabilization under freeze-thawing. Nonetheless, Ghosh et al. (2006) reported a stabilizing effect of unadsorbed proteins during frozen storage. When o/w emulsions were diluted with an aqueous dispersion of SC, the resultant emulsion ($\phi_m = 0.20$) was stable, but the amount of free oil was >30% when the dilution was carried out employing distilled water. According to these authors there are various mechanisms that could contribute to increase the freeze-thaw stability. First, the increased amount of unadsorbed proteins in aqueous phase may increase its viscosity or even form a transient gel that is able to mechanically resist the deformation exerted by expanding ice. Second, the nonadsorbed protein between two protein-covered surfaces would produce steric stabilization against coalescence. In our work, the values of AP % for SC emulsions at 1.0 and 2.0% w/v were 79.0 ± 2.3 and 71.3 ± 5.0, respectively (Table 1). The concentration of aqueous, nonadsorbed protein would be sufficient to stabilize the emulsions. In contrast, although the FW % was similar, at 0.5% w/v SC emulsions were destabilized by coalescence and flocculation, because it is probable that there is not enough protein to fully cover the interface created during the homogenization process. Moreover, the amount of aqueous protein (<0.1% w/v) would be too low to exert a stabilizing effect during frozen storage.

Besides, there are two main factors that could account for difference in freeze-thaw stability of emulsions prepared with NSI, DSI and sodium caseinate: a) the initial state of flocculation of the emulsion before freezing, and b) the loss of solubility of soy proteins at low temperature. With regard to the first factor, soy protein emulsions have a higher initial degree of flocculation than those prepared with SC sample (Table 1). These flocs in freshly prepared emulsions are not probably able to accommodate in the unfrozen aqueous channels between ice crystals, and hence, are rapidly destabilized by coalescence. Moreover, we can remark that in flocculated emulsions the spatial distribution of particles is not homogeneous. In the surroundings of droplets aggregates the local oil volume fraction is high, so that the stress due to expanding ice may rapidly lead to destabilization by coalescence and free oil separation after thawing. This would explain because the amount of free oil of emulsions prepared with 0.5% NSI and DSI (high initial values of FD %) were higher than 25%, unlike the SC emulsions in which the oiling off was <2% (Table 2). Then, NSI and DSI emulsions are highly destabilized by freeze-thawing process as consequence of their high initial FD % (Table 1). When protein concentration increases (0.5–2.0% w/v), a lower FD % was obtained for NSI and DSI emulsions, which decreased dramatically the amount of free oil (<2%). Moreover, at 2.0% w/v NSI and DSI emulsions showed marked differences in coalescence stability after freeze-thaw treatment, even though unfrozen emulsions have a similar particle size and negligible FD % (Table 1). While freeze-thawed NSI emulsions exhibited high CD % values and low values of FD %; the behavior of those prepared with DSI was exactly opposite (Table 2, Fig. 4). In the absence and presence of SDS, $D_{3,2}$ values of freeze-thawed NSI emulsions were 0.95 and 82.5 μm, respectively. In contrast, these values were significantly lower for DSI emulsions (0.49 and 1.52 μm). Based on these results, we can remark that denatured soy proteins provide a better stability to freeze-thawing.

The second factor that may help explain the differences in stability among the mentioned emulsions is the high tendency of native soy proteins to aggregate by effect of low temperature (Hashizume, Kariuchi, Koyama, & Watanabe, 1971; Wolf & Sly, 1967). It is known that the chilling and freezing of aqueous dispersions of soy storage proteins induce the aggregation of 11S globulin (Wolf & Sly, 1967). At subzero temperatures, this aggregation involve the formation of species of high molecular weight (15S + >15S fractions) by thiol/disulfide exchange reactions, due to that the aggregates are effectively dissociated in aqueous dispersions with addition of 0.01 M of β-mercaptoethanol (Hashizume et al., 1971). According results showed in Table 1, the proteins are distributed between the continuous aqueous phase (as nonadsorbed or bulk protein) and the interfacial film. During freezing, ice is formed from water molecules both in the bulk phase and interface, promoting the protein aggregation in both zones. Cortés-Muñoz et al. (2009), in the study of the freeze-thawing of submicron emulsions prepared with whey protein isolate, reported that droplet aggregation phenomena was probably promoted through protein aggregation at the o/w interface. This process of cold denaturation-aggregation of proteins could lead to loss of protein functionality when the emulsions are thawed (McClements, 2004; Thanasukarn et al. 2004). In order to try to confirm whether a relationship exists between the loss of protein solubility (PS %) and freeze-thaw stability of o/w emulsions, additional experiments of freeze-thawing of aqueous dispersions and emulsions were carried out. The increase of protein concentration (0.5–2.0% w/v) decreased the PS % of NSI aqueous dispersions: a protein precipitate was effectively observed after freeze-thawing. This behavior was not observed in aqueous dispersions prepared with DSI and SC whatever the protein concentration (Table 3). The effect of freeze-thawing on NSI solubility is more pronounced at

Table 3
Protein solubility (PS %) of aqueous dispersions prepared with native soy isolate (NSI), thermally-denatured soy isolate (DSI) and sodium caseinate (SC) at different protein concentration (0.5, 1.0 and 2.0% w/v). Samples were frozen at −20 °C for 24 h and thawed at 20 °C.

Sample	Sample concentration (% w/v)	PS (%)	
		Initial	After freeze-thaw treatment
NSI	0.5	95.9 ± 2.5	84.4 ± 3.7
	1.0	94.0 ± 1.7	83.0 ± 4.0
	2.0	93.2 ± 3.5	78.0 ± 1.4
DSI	0.5	97.3 ± 1.4	96.9 ± 0.9
	1.0	97.0 ± 0.9	96.2 ± 1.2
	2.0	96.1 ± 1.7	96.8 ± 0.8
SC	0.5	97.4 ± 1.4	97.4 ± 2.4
	1.0	97.3 ± 1.9	98.2 ± 1.7
	2.0	98.0 ± 0.9	98.8 ± 0.6

higher protein concentration, due to more molecules of 11S interact to form protein aggregates in the non-frozen aqueous phase. As was mentioned above, the thermal treatment at pH 7.0 and relatively low protein concentration induces the dissociation of 11S globulin and formation of soluble aggregates. The ability of aggregation of 11S by chilling and freezing is lost as a consequence of previous thermal denaturation. Hence, PS % of DSI aqueous dispersions was not significantly modified by freeze-thawing. In addition, the low tendency of aggregation of caseins at pH 7.0 is in accord with the high PS % of SC aqueous dispersions after freeze-thawing (Table 3). According to McClements (2004), proteins undergo conformational changes after adsorption in order to maximize the number of favorable interactions and minimize the number of unfavorable in their new environment. After of adsorption, extensive protein–protein interactions at interface may lead to the formation of an interfacial film, which provide protection against the droplet coalescence. The effective concentration of proteins is dramatically increased when they are adsorbed on the oil droplets during the homogenization process (Keerati-u-rai & Corredig, 2009). Transmission electron micrographs showed the presence of aggregated protein in bulk phase for freeze-thawed NSI emulsions. In addition, the structural characteristics of o/w interface appear to be clearly different respect to those prepared with DSI and SC samples (Fig. 5). Presumably, the freeze-thaw treatment negatively affects the integrity of interfacial film of NSI emulsions as a consequence of high tendency to cold aggregation of native storage globulins.

As was previously observed (Table 3), for aqueous dispersions prepared with 2.0% NSI, the loss of PS % was ~17%. In other experiment, NSI and DSI o/w emulsions were centrifuged at high-speed (18,000*g*, 30 min) and aqueous protein concentration (C_{aq}) was subsequently determined before and after freeze-thawing, according to experimental procedure mentioned in Section 2.2.7. For NSI emulsions, a decrease of ~17% of C_{aq} after freezing treatment (from 0.70 to 0.58% w/v) was effectively observed. This result would be consistent with the insoluble protein aggregates observed by electron microscopy observations (Fig. 5). In the opposite, C_{aq} values of fresh and freeze-thawed emulsions were similar (~0.56% w/v). This decrease of C_{aq} observed in NSI emulsion is in agreement with the loss of PS % of aqueous dispersion. As was mentioned above, Ghosh et al. (2006) reported a stabilized effect of aqueous protein of unadsorbed SC proteins on freeze-thaw stability of o/w emulsions. It is probably that unadsorbed native globulins proteins are not able to exert this stabilizing effect due to its high tendency to aggregation at subzero temperature.

We must remark that the differences in freeze-thaw stability of o/w emulsions prepared with NSI and DSI (especially at 1.0 and 2.0% w/v) are effectively observed when the fresh NSI sample (freeze-dried NSI sample is stored for a short time period before its utilization) was used. The aging of sample promotes the gradually loss of protein solubility of aqueous dispersions, which can affect dramatically the stability of o/w emulsions subjected to freeze-thawing. An observation made by us in a previous work (Palazolo & Wagner, 2007), provides some support. In that work, we utilized a freeze-dried NSI sample stored for one year at −20 °C. The loss of PS % in this sample (respect to fresh NSI) was approximately 15% due to aging. This result is not associated with a different composition of protein sample due to the soy isolates have similar composition. For initial emulsions prepared with this stored NSI at 2.0% w/v, we reported a FD % of 155 (significantly higher than those obtained in the present work, FD % = 18, Table 2). When this initial emulsion was stored at −20 °C for 24 h, a considerable instability was observed (free oil > 25%, CD % $>10^4$). Remarkably, DSI aqueous dispersions prepared from aged NSI at 2.0% w/v gave also very unstable o/w emulsions to freeze-thawing (data not shown). Based on these observations, it seems that the aging of NSI sample has a decisive influence on degree of flocculation of freshly prepared NSI and DSI emulsions, and their further response to freeze-thaw treatments.

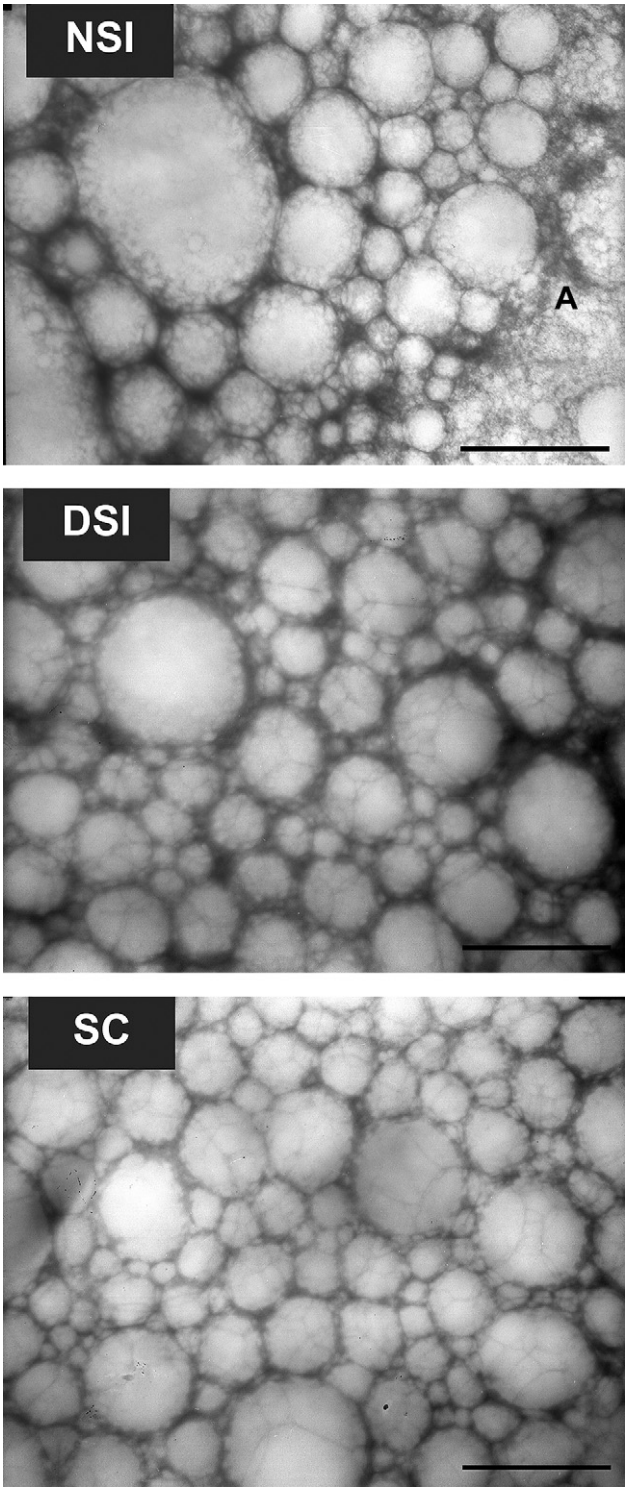

Fig. 5. Transmission electron micrographs of freeze-thawed o/w emulsions prepared with NSI, DSI and SC samples (2.0% w/v). For freeze-thawed NSI emulsion: A: protein aggregate. Freeze-thaw protocol was described in Section 2.2.5. Scale bar is 0.5 μm.

It is possible conclude that the thermal treatment of soy isolates improve the stability of o/w emulsions to freeze-thawing. Although the flocculation cannot be controlled, coalescence is almost totally

inhibited. This behavior is observed at high protein concentration where there is enough protein to cover the newly created interface during the homogenization and hence, the bridging flocculation of fresh emulsion is totally inhibited. Based on previous report (Palazolo & Wagner, 2007), the aging of sample also has a detrimental effect on freeze-thaw stability. As previously noted, the destabilization of emulsions during freeze-thawing was probably promoted by disruption or collapse of interfacial film as a consequence of protein aggregation in bulk phase and at the o/w interface. This effect is especially important in NSI emulsions subjected to freeze-thawing.

4. Conclusions

The treatment of freeze-thawing is highly destabilizing to many food emulsions. The maintenance of integrity of interfacial film in conditions where the water crystallizes is decisive to assure the preservation of the initial characteristics of emulsions after thawing or reheating. This study provides information on freeze-thaw stability of emulsions prepared with native and thermally-denatured soy proteins in comparison with sodium caseinate, a protein sample fully utilized as emulsifier. The increase of protein concentration for emulsions prepared with native and denatured soy isolate is a stabilizing factor against the storage at subzero temperature, where the aqueous phase crystallizes. At low protein concentration (0.5–1.0% w/v), the freeze-thaw stability of soy isolate-stabilized o/w emulsions were remarkably lower than those prepared with sodium caseinate. The initial degree of flocculation of soy isolate-stabilized o/w emulsions, related both with the previous thermal treatment of native soy isolate and the sample aging, seems to be a decisive influence on their freeze-thaw stability. At high protein concentration, where is enough protein to cover the interface during the homogenization, the emulsions prepared with denatured sample exhibited a good behavior due to the ability of protein aggregation of native globulins (especially, the 11S globulin) both in aqueous phase and at the interface is lost when the soy isolate is thermally treated. For this emulsion, the coalescence and oiling off is almost inhibited, but unlike the sodium caseinate-stabilized emulsions, the formation of new flocs during the storage at subzero temperatures cannot be totally controlled. In this work, sunflower oil was used as lipid phase and do not crystallizes during frozen storage. Since the oil crystallization can also affect the freeze-thaw stability of o/w emulsions, these results cannot generalize to any type of oil. In further works, we intend study the incorporation of other triglycerides composed by fatty acids of high saturation degree as lipid phase and some cryoprotectans (sugars, polyols, and soy oligosaccharides) in aqueous phase to evaluate their influence on freeze-thaw stability of soy protein-stabilized emulsions.

Acknowledgements

The authors of this article gratefully acknowledge the financial support of the ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica, PICTO-UNQ 2006 36473) and the Universidad Nacional de Quilmes (I+D Grant PUNQ 53/1007). Pablo A. Sobral is research fellow of Universidad Nacional de La Plata. Gonzalo G. Palazolo and Jorge R. Wagner are members of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina).

References

Anton, M., Beaumal, V., Brossard, C., Llamas, G., & LeDenmat, M. (2002). Droplet flocculation and physical stability of oil-in-water emulsions prepared with hen egg yolk. In M. Anton (Ed.), *Food emulsions and dispersions* (pp. 15–28). Kerala, India: Research Signpost.

Arai, S., & Watanabe, M. (1988). Emulsifying and foaming properties of enzymatically modified proteins. In E. Dickinson, & G. Stainsby (Eds.), *Advances in food emulsions and foams* (pp. 189–220). Essex, UK: Elsevier Applied Science Publishers Ltd.

Bamba, J., Cavé, G., Bensouda, Y., Tcholeroff, P., Puiseux, F., & Couarraze, G. (1995). Cryoprotection of emulsions in freeze-drying: freezing process analysis. *Drug Development and Industrial Pharmacy, 21*(15), 1749–1760.

Carrera Sánchez, C., & Rodríguez Patiño, J. M. (2005). Interfacial, foaming and emulsifying characteristics of sodium caseinate as influenced by protein concentration in solution. *Food Hydrocolloids, 19*, 407–416.

Castellani, O., Belhomme, C., David-Briand, E., Guérin-Dubiard, C., & Anton, M. (2008). The role of metal ions in emulsion characteristics and flocculation behaviour of phosvitin-stabilised emulsions. *Food Hydrocolloids, 22*, 1243–1253.

Cortés-Muñoz, M., Chevalier-Lucia, D., & Dumay, E. (2009). Characteristics of submicron emulsions prepared by ultra-high pressure homogenisation: effect of chilled or frozen storage. *Food Hydrocolloids, 23*, 640–654.

Cramp, G. L., Docking, A. M., Ghosh, S., & Coupland, J. N. (2004). On the stability of oil-in-water to freezing. *Food Hydrocolloids, 18*, 899–905.

Dickinson, E. (2006). Structure formation in casein-based gels, foams and emulsions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 288*, 3–11.

Dickinson, E., Golding, M., & Povey, M. J. W. (1997). Creaming and flocculation of oil-in-water emulsions containing sodium caseinate. *Journal of Colloid and Interface Science, 185*, 515–529.

Ghosh, S., & Coupland, J. N. (2008). Factors affecting the freeze thaw stability of emulsions. *Food Hydrocolloids, 22*, 105–111.

Ghosh, S., Cramp, G., & Coupland, J. N. (2006). Effect of aqueous composition on the freeze-thaw stability of emulsions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 272*, 82–88.

Hashizume, K., Kariuchi, K., Koyama, E., & Watanabe, T. (1971). Denaturation of soybean protein by freezing. Part I. *Agricultural Biological Chemistry, 33*, 449–459.

Keerati-u-rai, M., & Corredig, M. (2009). Heat-induced changes in oil-in-water emulsions stabilized by soy protein isolate. *Food Hydrocolloids, 23*, 2141–2148.

Kinsella, J. E., & Whitehead, D. M. (1988). Emulsifying and foaming properties of enzymatically modified proteins. In E. Dickinson, & G. Stainsby (Eds.), *Advances in food emulsions and foams* (pp. 163–188). Essex, UK: Elsevier Applied Science Publishers.

Markwell, M. A. K., Haas, S., Bieber, L. L., & Tolbert, N. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry, 87*, 206–210.

McClements, D. J. (2004). Protein-stabilized emulsions. *Current Opinion in Colloid and Interface Science, 9*, 305–313.

Mitidieri, F. E., & Wagner, J. R. (2002). Coalescence of o/w emulsions stabilised by whey and isolate soybean proteins. Influence of thermal denaturation, salt addition and competitive interfacial adsorption. *Food Research International, 35*, 547–557.

Molina, E., Papadopoulou, A., & Ledward, D. A. (2001). Emulsifying properties of high pressure treated soy protein isolate and 7S and 11S globulins. *Food Hydrocolloids, 15*, 263–269.

Nir, I., Feldman, Y., Aserin, A., & Garti, N. (1994). Surface properties and emulsification behaviour of soy proteins. *Journal of Food Science, 59*, 606–610.

Palanuwech, J., Poniteni, R., Roberts, R. F., & Coupland, J. N. (2003). A method to determine free fat in emulsions. *Food Hydrocolloids, 17*, 55–62.

Palazolo, G. G., Mitidieri, F. E., & Wagner, J. R. (2003). Relationship between interfacial behaviour of native and denatured soybean isolates and microstructure of o/w emulsions. Effect of salt and protein concentration. *Food Science and Technology International, 9*, 409–421.

Palazolo, G. G., Sorgentini, D. A., & Wagner, J. R. (2004). Emulsifying properties and surface behaviour of native and denatured whey soy proteins in comparison with other proteins. Creaming stability of o/w emulsions. *Journal of the American Oil Chemist's Society, 81*, 625–632.

Palazolo, G. G., Sorgentini, D. A., & Wagner, J. R. (2005). Coalescence and flocculation in o/w emulsions of native and denatured whey soy proteins in comparison with soy proteins isolates. *Food Hydrocolloids, 19*, 595–604.

Palazolo, G. G., & Wagner, J. R. (2007). Effect of freezing on stability of soy protein and sodium caseinate oil-in-water emulsions. In C. E. Lupano (Ed.), *Functional properties of food components* (pp. 33–56). Kerala, India: Research Signpost.

Puppo, M. C., Speroni, F., Chapleau, N., de Lambarellie, M., Añón, M. C., & Anton, M. (2005). Effect of high-pressure treatment on emulsifying properties of soybean proteins. *Food Hydrocolloids, 19*, 289–296.

Qi, Hettiarachchy, N. S., & Kalaphaty, U. (1997). Solubility and emulsifying properties of soy protein isolates modified by pancreatin. *Journal of Food Science, 62*, 1110–1115.

Relkin, P., & Sourdet, S. (2005). Factors affecting fat droplet aggregation in whipped frozen protein-stabilized emulsions. *Food Hydrocolloids, 19*, 503–511.

Roos, Y. H. (1995). *Phase transitions in foods*. San Diego: Academic Press, Inc.

Smulders, P. E., Caessens, P. W., & Walstra, P. (1999). Emulsifying properties of β-casein and its hydrolysates in relation to their molecular properties. In E. Dickinson, & J. M. Rodríguez Patino (Eds.), *Food emulsions and foams (interfaces, interactions and stability)* (pp. 61–69). UK: The Royal Society of Chemistry.

Sorgentini, D. A., Añón, M. C., & Wagner, J. R. (2002). Enhancement of hydration and surface properties of soy isolates by thermal and acid treatments. In M. Anton (Ed.), *Food emulsions and dispersions* (pp. 29–39). Kerala, India: Research Signpost.

Sorgentini, D. A., & Wagner, J. R. (1999). Comparative study of structural characteristics and thermal behavior of whey and isolate soybean proteins. *Journal of Food Biochemistry, 23*, 489–507.

Thanasukarn, P., Pongsawatmanit, R., & Mc Clements, D. J. (2004). Influence of emulsifier type on freeze-thaw stability of hydrogenated palm oil-in-water emulsions. *Food Hydrocolloids, 18*, 1033–1043.

Tornberg, E., Olsson, A., & Persson, K. (1997). The structural and interfacial properties of food proteins in relation to their function in emulsions. In S. E. Friberg, & K. Larsson (Eds.), *Food emulsions* (3rd ed., revised and expanded). (pp. 267–391). New York: Marcel Dekker.

Wolf, W. J., & Sly, D. A. (1967). Cryoprecipitation of soybean 11S protein. *Cereal Chemistry, 44*, 653–668.

Xiong, Y. L. (1997). Protein denaturation and functionality losses. In M. C. Erickson, & Y. C. Hung (Eds.), *Quality of frozen foods* (pp. 111–139). USA: Chapman & Hall.

Yamauchi, F., Yamagishi, T., & Iwabuchi, S. (1991). Molecular understanding of heat-induced phenomena of soybean protein. *Food Reviews International, 7*, 283–322.