Food Hydrocolloids 74 (2018) 115–123

Contents lists available at ScienceDirect

Food Hydrocolloids

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

Comparative behaviour of solutions and dispersions of amaranth proteins on their emulsifying properties

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ARTICLE INFO

Article history:
Received 17 May 2017
Received in revised form
25 July 2017
Accepted 28 July 2017
Available online 2 August 2017

Keywords:
Emulsions
Protein solutions
Protein dispersions
Amaranth

ABSTRACT

In the present work the effect of the presence of soluble and insoluble protein on the stability of oil-water emulsions prepared with amaranth protein isolates (API) was analyzed. For this purpose, four types of emulsions were prepared: API-pH2 and API-pH6.3 dispersions and solutions. At pH 2.0 the amaranth proteins present higher solubility, are denatured and partially hydrolyzed; while at pH 6.3 its solubility is lower and its structure is more similar to the native protein. The soluble proteins present in API-pH2 and API-pH6.3 reduce with equal intensity the interfacial tension. However, the proteins present in API-pH2 are adsorbed twice as fast as those present in API-pH6.3, with equal rearrangement rate at the oil/water interface. Both, solutions and dispersions of API-pH2 and API-pH6.3 allow the formation of oil-in-water emulsions. Flocculation phenomena are evident, particularly in the case of API-pH6.3 dispersions. The calculated creaming-flocculation constant demonstrates that stability of emulsions increase with protein concentration and with the decrease of pH. The behaviour of API-pH2 and API-pH6.3 solutions was similar to that corresponding to the dispersions discarding a negative effect of the insoluble protein on the emulsifying properties of amaranth proteins.

1. Introduction

According to prospective studies carried out by international organisations, during the next decades, the world population will continue to increase; and along with this, there will also be an increase in food demands, which will have to be fulfilled in a scenario of fresh water shortages, reduced areas of cultivable lands and profound climate changes (United Nations, 2013; Alexandratos & Bruinsma, 2012). In addition, there is also increasing awareness of the relationship between the type of foods people consume and their health status and well-being (Council of the European Union, 2007; Udenigwe & Aluko, 2012). In this context, alternative plant cultivations, such as those of amaranth, which have long been cultivated by the Inca, Mayans and Aztec peoples, emerges as an interesting alternative, especially for their ability to fix higher amounts of carbon dioxide, as every C4-type plant, for its agricultural features and the nutritional bioactive properties of is proteins (Janssen et al., 2016; Kiegel, 1994). The amaranth grains storage proteins have an excellent amino acid balance and are known to have antihypertensive, antioxidant, antithrombotic, antiproliferative, cholesterol lowering and immunomodulatory properties (Caselato-Sousa, & Amaya-Farfán, 2012; Fritz, Vecchi, Rinaldi, & Añón, 2011; Montoya-Rodriguez, Gomez-Favela, Reyes-Moreno, Millan-Carrillo, & Gonzalez de Mejía, 2015; Moronta, Smaldini, Docena, & Añón, 2016; Moronta, Smaldini, Fossati, Añón, & Docena, 2016; Orsini Delgado, Galleano, Añón,& Tironi, 2015; Quiroga, Barrio, & Añón, 2015; Sabbione, Scilingo, & Añon, 2015). One of the main drawbacks of functional foods is that its biologically active peptides do not always reach the target organs due to the sensitivity of such peptides to hydrolysis by gastrointestinal proteases.

Several studies have demonstrated that amaranth storage proteins also have good emulsifying, foaming, gelifying and film-forming properties, as well as a good water retention capacity (Avanza, Puppo, & Añón, 2005; Bolontrade, Scilingo, & Añón, 2013; Bolontrade, Scilingo, & Añón, 2016; Shevkani, Singh, Rana, & Kaur, 2014; Silva-Sánchez, González-Castañeda, De León-Rodríguez, & Barba de la Rosa, 2004; Ventureira, Martinez, & Añón, 2010, 2012a, 2012b).

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http://dx.doi.org/10.1016/j.foodhyd.2017.07.042
0268-005X/

Food emulsions have been proposed as bioactive compound transporters (Adjonu, Doran, Torley, & Agboola, 2014; McClemens, 2010). It has been demonstrated that protein hydrolysates, many of which are known to contain physiologically active peptides, can form emulsions; however, these emulsions are not stable; particularly when such hydrolysates contain peptides of low or very low molecular mass (Scherze & Muschiolik, 2001). In addition, there exist controversies about the deleterious effects that the presence of insoluble proteins may entail, particularly as regards the protein emulsifying properties.

It is well known that amaranth proteins present reduced solubility at pH values near the neutrality and under low ionic strength conditions (Bolontrade et al., 2013). These conditions are very frequent in the food industry and therefore, represent a limitation for the use of such proteins. Several studies have demonstrated that the solubility of these vegetal proteins can be increased by working at pHs lower than 4.5, a value that corresponds to the average isoelectric point (pI) of amaranth storage proteins (Bolontrade et al., 2013; Shevkani et al., 2014; Ventureira et al., 2010). Even though the latter is a possibility to increase the solubility, this strategy has limited use due to the slightly acidic or nearly neutral pH of foods.

Taking into account this restriction imposed by the protein solubility and, considering that amaranth proteins have the capacity to form and stabilise emulsions that could act as potential transporters of bioactive ingredients, the aim of this work was to analyse the effect of the insoluble protein content in the emulsifying properties of amaranth protein isolates at acidic pH (API-pH2) and at nearly neutral pH (API-pH6.3).

2. Materials and methods

2.1. Amaranth seeds and flour

Seeds of *Amaranthus hypochondriacus* were obtained from INDEAR (Instituto de Agrobiotecnología de Rosario, Argentina). The flour was obtained by grinding the seeds as described previously. Crude protein of flour was 18.7 ± 0.4% (dry basis); as determined by the Kjeldahl method (AOAC, 1984) using a factor of 5.85 (Scilingo, Molina Ortiz, Martinez, & Añón, 2002). The content of carbohydrate, lipids and ash were 68.2 ± 1.6% w/w, 7.4 ± 0.7% w/w and 3.2 ± 0.1% w/w, respectively (all results are expressed in dry basis).

2.2. Preparation of amaranth protein isolates (API)

API were obtained by alkaline extraction, pH 9.0, and isoelectric precipitation, pH 5.0, as previously described by Martínez and Añón (1996). The protein content of isolates was 84.7 ± 4.2% (Nx5.85) (dry basis), as determined by the Kjeldahl method. The isolates also contain 3.3 ± 0.3% w/w of ash and 10% w/w of carbohydrate, basically soluble fiber (results are expressed in dry basis).

2.3. Protein solubility

API were solubilized in phosphate buffer pH 2.0 (API-pH2) (0.052 M H_3PO_4, 0.048 M KH_2PO_4, 0.052 M NaCl, ionic strength 0.1) and pH 6.3 (API-pH6.3) (0.047 M KH_2PO_4, 0.013 M K_2HPO_4, ionic strength 0.1). Dispersions (total protein: soluble + insoluble fractions) of a protein concentration of 0.1% w/v were stirred for 1 h at room temperature. Dispersions were centrifuged at 15,000 × g for 15 min at 20 °C and the supernatants corresponded to the protein soluble fraction (API-pH2 and API-pH6.3 solutions). The protein content in the supernatant was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The protein solubility (PS%) was calculated as the ratio between the protein content in the supernatant (Ps) and the total protein content (Pt) determined by the Kjedahl method (N x 5.85).

$$PS\% = Ps \times 100/Pt \quad (1)$$

2.4. Differential scanning calorimetry (DSC)

Dispersions and solutions at 2% w/v of API-pH2 and API-pH6.3 were frozen at − 80 °C and lyophilized. Hermetically sealed aluminium pans were prepared to contain 10–15 mg of either lyophilized API-pH2 or API-pH6.3 dispersions or solutions in distilled water (20% w/v). A hermetically pan containing lyophilized API-pH7.5 was also prepared under the same conditions. The value of the enthalpy of this sample was selected as reference for the calculation of the degree of denaturation attained by API-pH2 and API-pH6.3. A double empty pan was employed as reference. Capsules were heated from 20 to 120 °C at a rate of 10 °C min^{-1}. DSC measurements were performed in a TA Q100 calorimeter (TA-Instruments, USA). The equipment was calibrated at a heating rate of 10 °C min^{-1} by using indium, lauric acid, and stearic acid (p.a.) as standards. The dry matter content of samples was determined by leaving the pans overnight in an oven at 105 °C and then weighed. The denaturation temperature (Td, °C) and the enthalpy of transition (ΔHd, J g^{-1} dry protein) were obtained by analyzing the thermograms with the Universal Analysis 2000 Software.

2.5. Electrophoresis

SDS-PAGE was carried out under reducing conditions according to Laemmli (1970) in stacking, resolving and spacer gels that contained 12, 4 and 10% w/v acrylamide respectively. Runs were performed in minislabs (Bio-Rad Hercules, CA, USA). Phosphorylase b (94 kDa), bovine serum albumin (67 kDa); ovoalbumin (43 kDa); carbonic anydrase (30 kDa); trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used as molecular markers.

2.6. Zeta-potential measurements

The zeta-potential values of API suspended in water at pH 2.0 and 6.3 were measured by a dynamic laser light scattering using a Nano Particle analyzer SZ-100 (Horiba Scientific Inc., UK) at 25 °C. The zeta-potential was determined by measuring the direction and rate of droplet movement in a well-defined electric field. 500 μL of diluted sample (0.05% w/v) was put in the electrophoresis cell.

2.7. Preparation of oil-water emulsions

Emulsions were prepared with either the total protein (API-pH2 and API-pH6.3 dispersions) or with the soluble fraction of API (API-pH2 and API-pH6.3 solutions). For dispersions, the sample concentration was 0.1, 0.4, 0.8 and 1.7% w/v and for solutions were 0.8 and 1.7% w/v of total proteins corresponding to: 0.7 and 0.2% w/v and 1.4 and 0.5% w/v of API-pH2 and API-pH6.3 respectively. Samples were suspended in the buffers described in Section 2.3, stirred 1 h at room temperature and then centrifuged at 7000 × g for 15 min at 20 °C. Emulsions were prepared by homogenizing 4 mL of refined sunflower oil and 16 mL of the protein dispersion or solutions (20% oil v/v) with an ULTRA-TURRAX T25 rotor/stator (Janke & Kunkel GmbH, Staufen, Germany) homogenizer at a rate of 20.000 rpm for 1 min, to produce coarse emulsions. Samples were then further homogenized with an ultrasound homogenizer (SONICS Vibra Cell VCX750) at a power level of 50%, applying pulses of 30 s each with the standard tip immersed 2/3 in a 28 mm

diameter glass. The glass was placed into an ice bath to reduce heating during homogenization.

2.8. Particle size distribution

The volume-weighted mean droplet size ($D_{4,3}$) of freshly prepared or stored (in refrigerator for 24 h) emulsions prepared with API-pH2 and API-pH6.3 dispersions or solutions were determined using a Malvern MasterSizer 2S, Malvern Instruments, Ltd, UK.

About 500 μL of sample was diluted to 600 mL of water and the pump speed in dispersion unit was set at 2000 rpm (Hydro 2000MU, Malvern Instruments, Worcestershire, UK). In order to measure the particle size distribution avoiding flocculation, 500 μL of the emulsions were poured in a tube containing 11.5 mL of 1% w/v SDS solution and the measurement was performed after 5 min. The diluted emulsions were recirculated in water until an obscuration level of 10–20% was reached. The variation of $D_{4,3}$ values at different times, in the presence or absence of SDS, was used to calculate the flocculation index (FI) and the coalescence index (CI), as described previously (Ventureira et al., 2012a).

$$FI = (D_{4,3\ t} - D_{4,3\ t\ +\ SDS}) / D_{4,3\ t\ +\ SDS} \quad (2)$$

$$CI = (D_{4.3\ t\ +\ SDS} - D_{4.3\ in\ +\ SDS}) / D_{4.3\ in\ +\ SDS}) \quad (3)$$

Where

$D_{4,3\ t}$ is the value of $D_{4,3}$ at any time
$D_{4,3\ +\ SDS}$ is $D_{4,3}$ measured in the presence of SDS
$D_{4.3\ in\ +\ SDS}$ is the initial value of $D_{4,3}$ measured in the presence of SDS.

2.9. Viscosity of emulsions

Emulsion viscosity was measured in a Controlled Stress Rheometer Haake RS 600 (Thermoelectron, Karlsruhe, Germany) using a plate–plate sensor system with a 1.0 mm gap between plates and shear rates from 1.0 to 500 s^{-1}. The apparent viscosity of each emulsion, prepared with API-pH2 and API-pH6.3 dispersions or solutions, was determined at 25 °C at a shear rate of 100 s^{-1}. Experimental flow curves were compared to Power's law model, which is the typical equation to characterize shear-thinning fluids

$$\tau = K.\ \gamma^{n} \quad (4)$$

where

τ is the shear stress (Pa)
K is the consistency index (Pa.s)
γ is the shear rate (s^{-1})
n is the flow index

2.10. Interfacial tension and rheological properties of the oil-water interface

The dynamic interfacial tension (γ) measurement at the oil/water interface was carried out using an automated drop tensiometer (Tracker IT-Concept, Saint-Clémenttes Places, France) with a rising oil drop in the aqueous medium. The axial symmetric drop is formed to reach a volume of 8 μL, which represents an interfacial area of approximately 18 mm^2. The aqueous medium consisted of API-pH2 and API-pH6.3 solutions at a concentration of 0.1% w/v. Measurements were performed at room temperature (20 °C) and for 7200 s. Periodical sinusoidal compressions and expansions of the drop volume were made. The surface dilatational modulus (E), its elastic (E_d) and viscous (E_v) components, and the phase angle (θ) were derived from the change in the interfacial tension ($d\gamma$) resulting from a small change in the relative interfacial area (dA/A_0) in the linear region of viscoelasticity. The oscillation frequency was 0.2 Hz after 7200 s (equilibrium state). The area variations of the droplet surface were+/−3% of the initial interfacial value, which fell within the linear region of viscoelasticity.

From them and using the kinetic model proposed by Panizzolo (2005), the rate constants were obtained for the adsorption and reordering processes. The equation used was:

$$\gamma_t = A_a e^{-k_a t} + A_r e^{-k_r t} + \gamma_e \quad (5)$$

where

k_a and k_r (s^{-1}) are first order rate constants for the processes of adsorption and rearrangement of proteins at the oil-water interface respectively
A_a, A_r ($mN\ m^{-1}$): are the amplitude parameters.
γ_e ($mN\ m^{-1}$): surface tension of equilibrium

2.11. Global emulsion stability

The stability of the emulsions was determined with a vertical scan analyzer Quick Scan (Beckman–Coulter inc., USA). Samples were loaded into a cylindrical glass measurement cell, and the profiles of backscattering percentage (%BS) were immediately monitored all along the cell. The emulsion prepared at 0.1% w/v. API was monitored every 3 min for 1 h and for the rest of the emulsions every day for 10 days as a function of the sample height (total height = 60 mm). Cells were stored at 4 °C until the time of measurement. The creaming-flocculation and coalescence-flocculation kinetics were obtained following the variation mean %BS as a function of time in the lower part (10–15 mm height) and the upper part (40–50 mm height) of the cell, respectively. The creaming-flocculation constant ($k_{0.1}$) was determined as proposed by Palazolo, Sorgentini, and Wagner (2005) and it was defined as $k_{0.1}$ and was defined as:

$$k_{0.1} = (\%\ BS_{in} \times t_{0.1})^{-1} \quad (6)$$

where $\%BS_{in}$ is the initial mean value of backscattering; $t_{0.1}$ is the time is the time where the mean $\%BS_{in}$ value decreases 10% respect of its initial value ($\%BS = 0.9\%\ BS_{in}$). Mean values of %BS were determined in the lower part of the tube. The increase of $k_{0.1}$ suggests a decrease of the emulsion stability.

2.12. Statistical analysis

Data were the mean of at least two independent assays. Results are reported as mean+/-standard deviation. Statistical analysis was carried out by the GraphPad Prism 6 (GraphPad Prism Inc., USA). Analyses of variance were conducted. Differences between the sample means were analyzed by Tukey Test using $\alpha = 0.05$.

3. Results and discussion

3.1. Characterization of proteins present in API-pH2 and API-pH 6.3 dispersions and soluble fractions

Based on previous results obtained in our laboratory, two experimental conditions were chosen to obtain amaranth proteins.

These conditions allowed obtaining two protein preparations that had structurally marked differences. API samples were dispersed in phosphate buffer at pH 2.0 and pH 6.3 and under low ionic strength conditions, thus obtaining the API-pH2 and the API-pH6.3 dispersions that were made up of a mixture of both soluble and insoluble proteins. After centrifugation, the corresponding solutions (soluble protein fractions) were obtained. Results indicated that, under low ionic strength conditions, amaranth storage proteins were much more soluble at pH 2.0 than at pH 6.3. PS% values were 84.0 ± 5.8 and 28.9 ± 1.4% w/v, respectively, in agreement with previous results obtained in our laboratory (Bolontrade et al., 2013).

Results obtained by DSC indicated that those proteins that were dispersed at pH 2.0 were completely denatured, whereas the ones dispersed at pH 6.3 were found to be only partially unfolded. The denaturation ΔH_d values for the API-pH 6.3 corresponding to the dispersion was 6.0 ± 0.3 J g^{-1} dry protein. The ΔH_d value for the API dispersed at pH 7.5 was 7.6 ± 0.9 J g^{-1} dry protein and, taking into account that under the latter conditions, proteins were in the native state in the sample, it can be deduced that the denaturation percentages at pH 6.3 were 22% for the proteins present in the dispersion. Both the precipitate obtained by centrifugation from the API-pH6.3 dispersion and the corresponding soluble fraction had ΔH_d value equivalent to that of the total dispersion indicating a similar distribution of partially denatured proteins in both fractions (results not shown).

In the electrophoretic runs performed under reducing conditions (data not shown), total dispersions and solutions (API and API-pH 6.3) presented the typical profiles corresponding the amaranth storage proteins made up of polypeptides derived from 7S (66.0 ± 1.0; 52 ± 1.1; 37.9 ± 0.3; 35.1 ± 0.5 10.8 ± 0.4 kDa), 11S (38–31.5 ± 0.5, 27.1–20.3 ± 0.6 kDa); P-storage globulin (34.2–32.0 ± 0.7; 22–20 ± 0.2 kDa), albumins and high molecular mass protein aggregates (Martinez, Castellani, & Añón, 1997). As for the total dispersion and the solution of API-pH2, and in line with the results published by Bolontrade et al. (2013), the profiles presented a lower proportion of polypeptidic species of high molecular mass, particularly, aggregates and small amounts of 66, 43, 25 and 21 kDa together with species smaller than 10 kDa.

According to the determined zeta potential values, the average electric charge of the proteins composing the API-pH2 was positive (31.3 ± 2.2 mV), while the corresponding to API-pH 6.3 was negative (−37.8 ± 1.7 mV). These results are expected considering the average isoelectric point reported for amaranth proteins (Konishi, Orikawa, Oku, Azumaya & Nakatani, 1991).

The results show that at pH 6.3, a value closer to the mean pI of the proteins present in API than pH 2.0, there is a drastic reduction of PS%. However, the proteins present in both the soluble and insoluble fractions largely retain their native conformation and the surface charge shows an important negative value. At pH 2.0 the results obtained for physicochemical and structural properties are in agreement with those previously obtained in our laboratory (Bolontrade et al., 2013; Ventureira et al., 2010, 2012a, 2012b). The acidic medium causes a complete protein unfolding and the dissociation of their quaternary structure due to the generation of a net positive charge. In addition, proteins are also partially hydrolyzed by a protease that is present in amaranth grains, which becomes activated at acidic pH only (Sabbione, Ibañez, Martinez, Añón & Scilingo, 2016; Ventureira et al., 2010, 2012a, 2012b). The latter partial hydrolysis leads to a reduction in the molecular mass of some polypeptides. Taken together, these factors lead to a greater PS% thus reverting one of the main drawbacks of amaranth storage proteins: their relatively low solubility. Undoubtedly, the decrease in the molecular mass that occurs in amaranth proteins at pH 2.0 - dissociation and partial hydrolysis - is most important that the average electric charge equality exhibited with the proteins at pH 6.3; differences in charge distribution and/or effects associated with the different molecular flexibility of amaranth proteins at these pHs cannot be ruled out.

3.2. Interfacial behaviour of soluble proteins at the oil/water interface

The reduction of the oil-water interfacial tension caused by API-pH2 and API-pH6.3 was determined. Such reduction in the interfacial tension can be assessed through: a) the diffusion towards the interface; b) the adsorption to the interface and 3) the unfolding of adsorpted proteins. All these three steps are known to occur simultaneously (Tornberg, Granfeldt, & Hakanson, 1982). In order to monitor these steps, the first order model described by Panizzolo (2005) was employed. Table 1 shows the rate constants corresponding to protein adsorption and rearrangement in the interface (k_a and k_r), as well as the surface tension amplitude parameters corresponding to the different conformational states of protein adsorption, re-arrangement and equilibrium in the interface (A_a, A_r and γ_e). Peptides and proteins present in the API-pH2 and API-pH6.3 caused a decrease in γ_e to reach values of 5.9 ± 0.3 and 6.6 ± 1.1 mN m^{-1}, respectively. It is evident that the main difference between the soluble proteins of API-pH2 and API-pH6.3, as regards the kinetics of modification of the surface tension, does not lie in the equilibrium values, for no significant differences were found between them, but in the rate at which such change occurs. The proteins present in API-pH2 became adsorbed twice times faster than the ones present in API-pH6.3 according to the k_a values obtains (Table 1). No differences were found in the protein rearrangement velocities in the interface being the k_r values obtained greater than those corresponding to k_a. According to the structural differences between the proteins present between both samples, previously discussed, it would be expected that API-pH2 proteins required a lower degree of rearrangement than those of API-pH6.3. Perhaps the partial denaturation reached at this last pH is enough to achieve a good anchorage at the interface. The decrease in the interface free energy caused by protein adsorption and re-arrangement phenomena was estimated by means of the amplitude parameters A_a and A_r, respectively. Only significant differences in the interface free energy caused by the adsorption of the protein were found, being smaller the one corresponding to the proteins present in API-pH2.

The composition and structure of the adsorpted proteins in the interface is reflected in the dilational viscoelastic behaviour, which is the factor that determines the stability of the resulting emulsion (Maldonado-Valderrama et al., 2008). The results shown in Table 1 indicate that the behaviour of the resulting interfaces corresponding to API-pH2 and API-pH6.3 is basically elastic, being the E_d and E values 34 and 79 times higher than those corresponding to E_v in API-pH2 and API-pH6.3. The angle of phase difference tangents

Table 1
Interfacial behaviour of API-pH2 and API-pH6.3 solutions, 0.1% w/v, at the oil/water interface.

	API pH 2.0	API pH 6.3
k_a (s^{-1})	$8\ 10^{-4} \pm 2\ 10^{-4a}$	$4\ 10^{-4} \pm 0^{b}$
k_r (s^{-1})	$8.4\ 10^{-3} \pm 2.8\ 10^{-3a}$	$7.6\ 10^{-3} \pm 1.2\ 10^{-3a}$
A_a (mN/m)	2.4 ± 0.5^{a}	4.2 ± 1.2^{b}
A_r (mN/m)	$3.7 \pm 0{,}9^{a}$	2.8 ± 0.6^{a}
γ_{eq} (mN/m)	$5.9 \pm 0{,}2^{a}$	6.6 ± 1.1^{a}
E_d (mN/m)	$30.6 \pm 0{,}6^{a}$	23.9 ± 1.9^{b}
E_v (mN/m)	0.9 ± 0.9^{a}	0.3 ± 0.0^{a}
E (mN/m)	30.6 ± 0.7^{a}	23.9 ± 1.8^{b}

Mean values with different lowercase letters in the same row indicate significant differences between samples at different pH ($\alpha < 0.05$, Tukey test).

was found to be 0.03 and 0.01, respectively. A similar rheological behaviour was described by Ventureira et al. (2012a) for amaranth protein isolates at pH 2.0 and pH 8.0; however, the value reached by the complex dilational modulus at pH 2.0 was six times higher than the one reported herein. Besides, these authors reported similar behaviours at pH 8.0 and pH 6.3. The differences between the results reported by Ventureira et al. (2012a) and those presented in this work might be ascribed to the different experimental conditions employed in both works, particularly as regards frequencies (0.02 and 0.2 Hz, respectively) and protein concentrations (0.01 and 0.1% w/v, respectively).

Results presented in this work are in line with the observations indicating that the surface elasticity is lower for flexible proteins than for globular ones (Lucassen-Reynders, Fainerman, & Miller, 2004). It must also be borne in mind that in API-pH2, proteins are completely unfolded, whereas at pH 6.3 the degree of unfolding is significantly lower, being their conformation mainly globular.

3.3. Emulsifying properties

3.3.1. Particle size distribution of oil/water emulsions

In order to evaluate the influence of the quality and proportion of soluble and insoluble fractions as tensioactive agents, oil-water emulsions were prepared with dispersions and solutions of API-pH2 and API-pH6.3 and different properties were analyzed. The particle size analysis showed monomodal distributions for all the samples assayed (dispersions and solutions of API-pH2 and API-pH6.3) (Figs. 1 and 2). In the case of dispersions (soluble + insoluble protein) four protein concentrations were analyzed (0.1, 0.4, 0.8 and 1.7% w/v), in the figures only the particle size distributions obtained at 0.8 and 1.7% w/v of total proteins were shown. For dispersions the presence of SDS caused a decrease in particle size and this effect was stronger for API-pH6.3 and at low protein concentrations (Fig. 1 panel C and Fig. 3 panels A and B), evidencing a higher degree of flocculation. It is likely that the latter phenomenon be due to a higher association capacity of the proteins present in API-pH6.3, particularly at high protein concentration, and/or to a lower solubility as compared with those present in API-pH2. In the case of the highest protein concentration, the $D_{4,3}$ values obtained with and without SDS were found to be equivalent for API-pH2 and API-pH6.3 ($D_{4,3}$ values were pH2.0: 20.4 ± 0.1 μm, without SDS and 1.2 ± 0.0 μm, with SDS; pH 6.3: 18.1 ± 1.8 μm, without SDS and 0.9 ± 0.0 μm, with SDS) (Fig. 3 panels A and B). The flocculation index (FI) values obtained for the different concentrations employed for the initial emulsions were (Table 2): 1.2, 6.6, 7.1 and 18.2 for API-pH2 and 0.1, 6.5, 10 and 18.9 for API-pH6.3 at 0.1, 0.4, 0.8 and 1.7% w/v total protein. The lowest proportion of protein employed (0.1% w/v) was insufficient to cover the created area and coalescence occurred during emulsification, which led to the formation of larger drops in the emulsion.

Emulsions that were prepared with the soluble fractions of API-pH2 and API-pH6.3 displayed a lower degree of flocculation (Fig. 2) than the corresponding dispersions (Fig. 1). In this case only two different protein concentrations were used. The FI determined for the initial emulsions were 5.2 and 6.5 for 0.7% and 1.4% w/v soluble protein from API-pH2 and 2.1 and 5.6 for 0.2% and 0.5% w/v soluble protein from API-pH6.3 (Fig. 3 panels C and D). These soluble protein concentration values correspond to the ones present in 0.8 and 1.7% w/v total protein. Should be noted, the particle size obtained in the presence of SDS was equivalent to that obtained with the dispersions containing both soluble and insoluble protein which would indicate that at least some of the protein that remains in the insoluble fraction has tensioactive properties, thus contributing to reduce the particle size (Fig. 3) or that the presence of the insoluble fraction does not affect the tensioactive capacity and consequently, the particle size in the emulsions.

In order to investigate the association capacity of the amaranth proteins the rheological properties of emulsions prepared with API-pH2 and API-pH6.3 dispersions were assessed at 1.7% w/v of protein. Results showed that, in both cases, the rheological behaviour corresponded to that of a pseudoplastic fluid (API-pH2: ηapp = 20.3 ± 0.1 mPa, n = 0.64 ± 0.05 and K = 0.08 ± 0.02; API-pH6.3: ηapp = 111 ± 4.8 mPa, n = 0.60 ± 0.02 and K = 0.26 ± 0.05). The dispersion of API-pH6.3 showed the highest viscosity and consistency index, besides at low rate gradients showed a characteristic overshoot of the presence of structures in the sample.

In the case of API-pH2 and API-pH6.3 solutions the viscosity values were found to be similar or lower than those corresponding to dispersions particularly in the case of API-pH6.3 where it

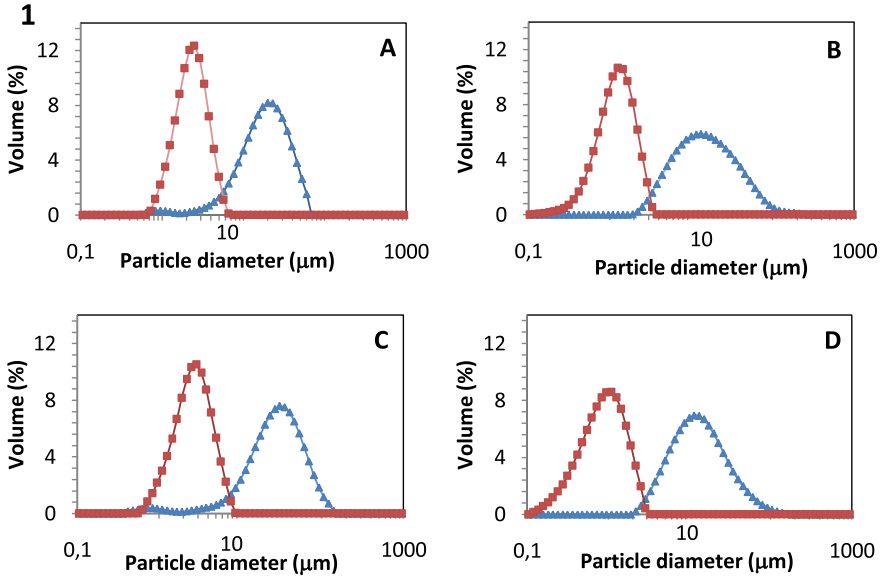

Fig. 1. Particle size distributions of oil/water emulsions prepared with API-pH2 and API-pH6.3 dispersions at 0.8 (panels A and C) and 1.7 (panels B and D) % w/v with SDS (-■-) and without SDS (-▲-).

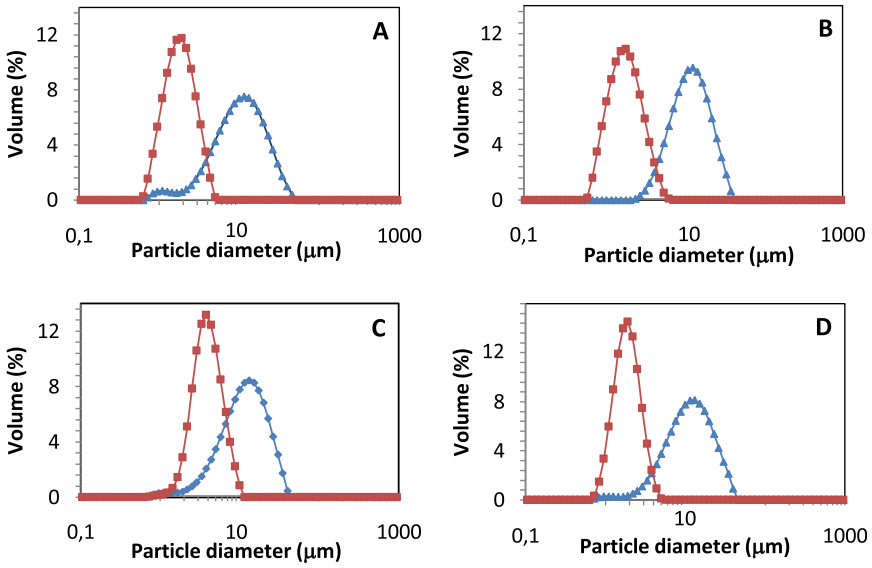

Fig. 2. Particle size distributions of oil/water emulsions prepared with API-pH2 solutions at 0.8 and 1.4% (panels A and C) and API-pH6.3 solutions at 0.2 and 0.5 (panels B and D) % w/v with SDS (-■-) and without SDS (-▲-).

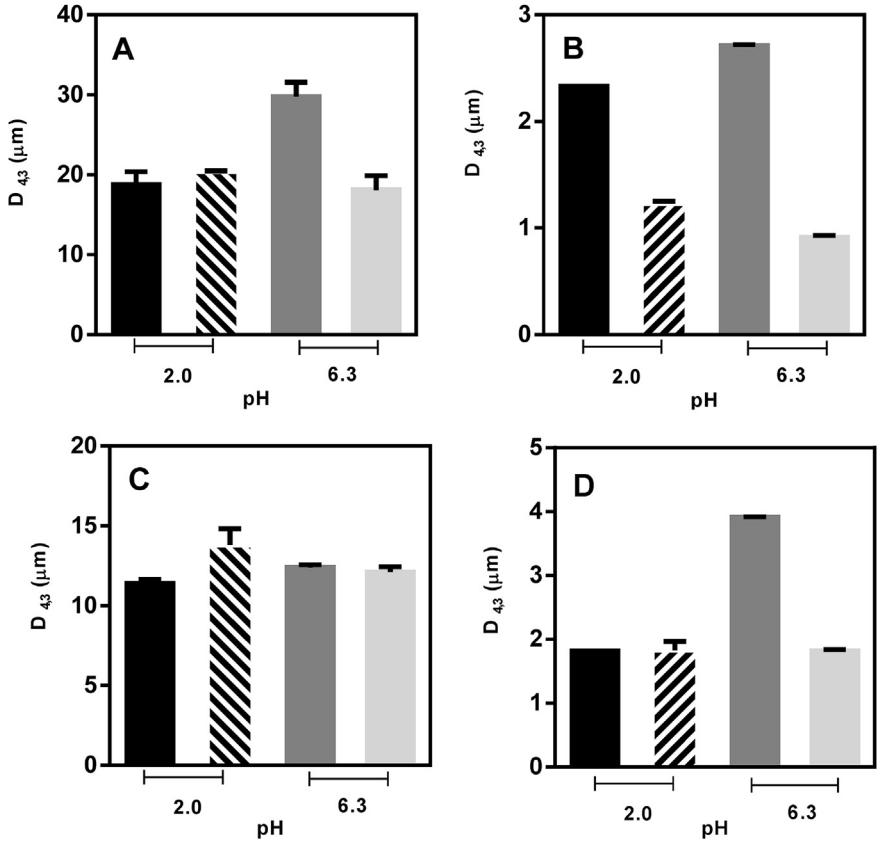

Fig. 3. Volume-weighted mean diameters ($D_{4,3}$) values corresponding to API-pH2 and API-pH6.3 dispersions (0.8 and 1.7% w/v protein concentrations) without SDS (panel A) and in presence of SDS (panel B) and API-pH2 and API-pH6.3 solutions (0.7 and 1.4 and 0.2 and 0.5% protein concentrations, respectively) without SDS (panel C) and in presence of SDS (panel D).

approaches to a Newtonian behaviour (API-pH2: ηapp = 18.6 ± 1.8 mPa, n = 0.63 ± 0.02 and K = 0.09 ± 0.02; API-pH6.3: ηapp = 9.1 ± 0.3 mPa, n = 0.96 ± 0.13 and K = 0.21 ± 0.01). Again a concordance between the rheological properties of proteins and their ease of flocculation is observed.

3.3.2. 2 Global stability and apparent viscosity on oil/water emulsions

The stability of the emulsions prepared with the four previously indicated total protein concentrations (0.1, 0.4, 0.8 and 1.7% w/v) were analyzed. Fig. 4 only shows the creaming kinetics

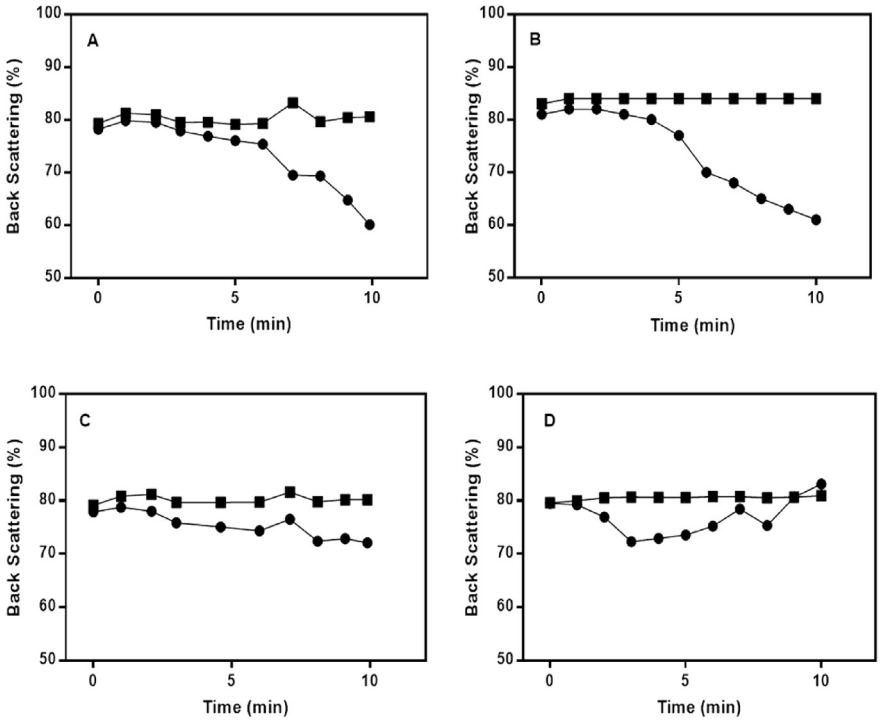

Fig. 4. Variations of the back scattering values (BS%) as a function of time for: A) API-pH2 dispersion 0.8%; B) API-pH2 dispersion 1.7%; C) API-pH6.3 dispersion 0.8% and D) API-pH6.3 dispersion 1.7%. The selected zones of the measurement were: 10–15 mm (creaming-flocculation kinetics (-●-) and 40–50 mm (flocculation-coalescence kinetics (-■-).

corresponding to the emulsions obtained with the dispersions of API-pH2 and API-pH-6.3 at 0.8 and 1.7% w/v protein concentrations. All the emulsions prepared with API-pH2 were highly stable to creaming and coalescence, except for those prepared with 0.1% w/v protein (results not shown). As stated above, it is evident that this protein concentration is insufficient to cover the drop area formed under these experimental conditions, therefore, coalescence occur during the emulsion formulation. This phenomenon led to the formation of larger drops, both in the presence and absence of SDS. At higher total protein concentrations (soluble and insoluble), that is 0.4, 0.8 and 1.7% w/v a decrease in the BS% was detected in the tube bottom; this phenomenon accounts for the drops migration towards the upper portion of the tube. Besides, this migration was not accompanied, at least throughout the time evaluated; by any evident creaming as evidence by the fact the BS values obtained in the upper part of the tube remained unchanged throughout the experiment. The latter phenomenon could be ascribed to the insufficient migration of drops to increase the BS in the upper part of the tube; probably due to a low migration rate or to the simultaneous presence of creaming and coalescence phenomena. It is highly probable that the high protein concentration present in the continuous phase form a flocs network that slows down the creaming process.

At 0.4% w/v, the decrease of BS started 2 h after the emulsion was prepared, reaching a decrease of a 60%, with respect to the initial value, after 10 days of storage at 4 °C. In the case of the higher protein concentrations, the decrease of BS was detected 5 days after the emulsion was prepared and, after 10 days of storage, a reduction of 30 and 20% had occurred.

The creaming-flocculation constant $K_{0.1}$ was calculated for all the analyzed samples. For API-pH2 dispersions, $k_{0.1}$ decreased with the total protein concentration (soluble + insoluble), and such values were 1.3 ± 0.1, 0.7 ± 0.1, 0.002 ± 0.000 y 0.002 ± 0.002 d^{-1}, for 0.1, 0.4, 0.8 and 1.7% w/v total protein, respectively.

The emulsions prepared with API-pH6.3 dispersion displayed a similar behaviour to that described above. At the lowest concentration assayed (0.1% w/v total protein), a decrease in the BS value corresponding to the lower part of the tube was detected after 10 min, to reach values of around 10% after 20 min. When higher total protein concentrations were employed, some changes started to occur after 1.5–2 days after the emulsion preparation, and the beginning of creaming in the upper part of the tube became evident by day 5–6.

For these emulsions, $k_{0.1}$ values were 3.2 ± 0.0, 1.4 ± 0.1, 0.012 ± 0.002 and 0.005 ± 0.002 d^{-1}; for the same total protein concentration range (0.1, 0.4, 0.8 and1.6% w/v).

The calculated $k_{0.1}$ values demonstrate that there is a direct relationship between emulsion stability and protein concentration, whereas such stability proved to have an inverse relationship with pH.

Table 2 shows the IF and IC values calculated from the previously obtained $D_{4,3}$ values (Figs. 1 and 2), after 1 day of emulsions preparation with dispersions of API-pH6.3 and API-pH2. The results show an increase of the IF with the total protein concentration present in the emulsion, particularly for the two higher concentrations of API-pH6.3. The IC values remained very close to zero for all the emulsions tested. Apparently the increased stability to creaming-flocculation of these emulsions would be associated with the ability to form a network of flocs which would delay both the creaming and the coalescence of the droplets.

Upon analysing the stability of emulsions prepared with the soluble fraction API-pH2 and API-pH6.3 (Fig. 5), the behaviour was equivalent to that of emulsions prepared with total protein (soluble + insoluble).

For API-pH2 at the highest protein concentration (1.4% w/v soluble protein, which corresponds to 1.7% w/v total protein) a higher stability was obtained, as compared with emulsions prepared only with soluble protein. The emulsions prepared under

Table 2
Volume-weighted mean diameter ($D_{4,3}$) in the presence of SDS, flocculation index (IF) and coalescence index (IC) values for emulsion prepared at APIpH2 and APIpH6.3 dispersions and solutions with different protein.

Parameter	dispersion	$D_{4,3}$ (SDS)		IF		IC	
		initial	1 day	initial	1 day	initial	1 day
Protein (% w/v)	pH						
0.1	pH 2.0	10.1 ± 6.3[a]	10.5 ± 0.1[a]	1.2[a]	1.1[a]	–	0.04[a]
0.1	pH 6.3	30.4 ± 0.0[b]	30.3 ± 0.1[b]	0.1[b]	0.5[b]	–	0[a]
0.4	pH 2.0	2.7 ± 0.1[a]	2.7 ± 0.0[a]	6.6[a]	6.0[a]	–	0.02[a]
0.4	pH 6.3	4.5 ± 0.1[b]	4.7 ± 0.10[b]	6.5[a]	6.4[b]	–	0[a]
0.8	pH 2.0	2.3 ± 0.0[a]	2.3 ± 0.0[a]	7.1[a]	6.1[a]	–	0[a]
0.8	pH 6.3	2.7 ± 0.0[b]	2,7 ± 0.1[b]	10[b]	9.9[b]	–	0[a]
1.7	pH 2.0	1.2 ± 0,0[a]	1.1 ± 0.1[a]	18.2[a]	18.3[a]	–	0[a]
1.7	pH 6.3	0.9 ± 0,0[a]	0.9 ± 0.0[a]	18.9[a]	24.8[b]	–	0[a]
Protein (% w/v)	solution	initial	7 days	initial	7 days	initial	7 days
	pH						
0.7	pH 2.0	1.8 ± 0.0[a]	3.2 ± 0.1[a]	5.2[a]	3.0[a]	–	0.8[a]
0.2	pH 6.3	3.9 ± 0.0[b]	4.3 ± 0.1[b]	2.1[b]	1.8[b]	–	0.1[b]
1.4	pH 2.0	1.8 ± 0.1[a]	1.7 ± 0.0[a]	6.5[a]	5.5[a]	–	0[a]
0.5	pH 6.3	1.8 ± 0.0[a]	2.0 ± 0.1[a]	5.6[b]	4.0[b]	–	0.1[a]

For samples at the same protein concentration the mean values with different lowercase letters in the same column indicate significant differences between samples at pH 2.0 and 6.3 ($\alpha < 0.05$, Tukey test).

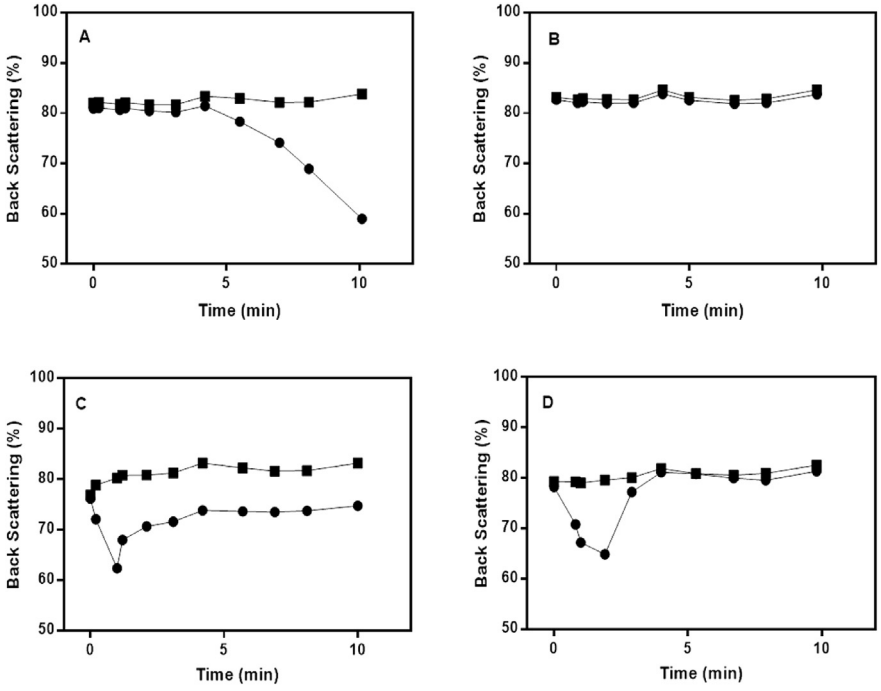

Fig. 5. Variations of the back scattering values (BS%) as a function of time for: A) API-pH2 solution 0.7%; B) API-pH2 solution 1.4%; C) API-pH6.3 solution 0.2% and D) API-pH6.3 solution 0.5%. The selected zones of the measurement were: 10–15 mm (creaming-flocculation kinetics (-●-) and 40–50 mm (flocculation-coalescence kinetics (-■-).

these conditions were stable up to 25 days after quiescent storage. In this case, the $k_{0.1}$ value could not be calculated, since a 10% decrease in the BS value was not achieved at the end of the experiment.

In the case of emulsions prepared with API-pH6.3 at 0.5% w/v soluble protein (equivalent to 1.7% w/v total protein), results were similar to those indicated above. Under these experimental conditions, the $k_{0.1}$ value was 0.014 ± 0.001 d^{-1}, which is a value that was higher than the one obtained with the total protein dispersion, thus indicating that the emulsions prepared with total proteins were more stable.

Emulsions prepared only with the soluble fraction showed an earlier change in the decrease of BS in the upper part of the tube.

Emulsions prepared with the soluble fractions of API-pH2 and API-pH6.3 present, after 7 days of storage, lower FI than those corresponding to dispersions, after 1 day of storage (Table 2). The CI values after 7 days of storage were also low.

The lower capacity of the soluble proteins to form a network of flocs would allow a greater mobility of the droplets through the emulsions and would affect the rate of creaming and coalescence.

For emulsions prepared with API-pH6.3, graphs of BS variation as a function of tube length evidenced the occurrence of water loss from the emulsion. This phenomenon was observed at low concentrations of total protein as well as at both concentrations of

soluble protein (Fig. 5). These findings suggest that the emulsion protein matrix was not able to retain the whole aqueous phase in the flocs network. For emulsions prepared with API-pH2, this effect was not detected in any case, neither with emulsions prepared with total nor with soluble protein.

The results obtained show that the coexistence of soluble and insoluble proteins in the aqueous phase of oil-in-water emulsions formulated with amaranth proteins does not alter their emulsifying capacity and stability and that it is possible to obtain very stable emulsions at high protein concentration suitable for use them as transport of different component as bioactive component.

4. Supporting information description

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, Argentina), Project PICT-2012-0937.

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