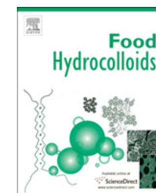
Food Hydrocolloids 24 (2010) 551–559

Contents lists available at ScienceDirect

Food Hydrocolloids

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



Stability of oil: Water emulsions of amaranth proteins. Effect of hydrolysis and pH

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ARTICLE INFO

Article history:
Received 8 July 2009
Accepted 23 December 2009

Keywords:
Amaranth proteins
Emulsion
Hydrolysis
Medium pH
Stability
Backscattering

ABSTRACT

In this contribution we have determined the effect of limited enzymatic hydrolysis on the emulsifying capacity of amaranth proteins. The action of enzyme (alcalase and trypsin) and the pH of the continuous phase of the oil/water emulsion (pH 2.0, 6.3 and 8.0) were the variables analyzed. The results obtained show that amaranth protein isolates, AI, contain proteins species capable of forming and stabilizing emulsions, mainly at acidic pH (2.0) and to a lesser extent at pH 8.0. While the emulsions obtained are sensitive to creaming and flocculation, they do not undergo destabilization by coalescence. The emulsions prepared from proteins subjected to low grade trypsin hydrolysis (TH2.2) are sensitive to creaming - flocculation, whereas alcalase-hydrolyzed proteins (AH1.7 and AH9.5) exhibited a significant destabilization by creaming, flocculation and coalescence, mainly at pH 6.3. The effect of the pH of the aqueous phase was determining on the emulsion stability beside the structural and physicochemical characteristics of protein species utilized as tensioactive. At acidic pH (pH 2.0) the unfolding and charge of polypeptides and the capacity of form a viscoelastic film at the interface were essential while at alkaline pH (pH 8.0) the balance among high and low molecular mass protein species and flexibility of the molecule fixed the emulsions properties.

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1. Introduction

There is an increasing demand of new food products with high nutritional quality. Protein isolates represent an interesting ingredient for such food formulations to which they must contribute not only with good nutritional properties, but also with suitable functional properties.

Emulsification is a functional property widely utilized in the food industry; many foods are totally o partially emulsified like mayonnaise, cream, sauces, desserts, comminuted meat products and some beverages (Dickinson & Stainsby, 1982; Friberg & Larsson, 1997; Jaynes, 1983, chap. 6; Swaisgood, 1996, chap. 14; McClements, 1999). The most important emulsifiers in such foods are proteins and low molecular weight surface active molecules (lipids, phospholipids) (Lizarraga, Pan, Añón, & Santiago, 2008). Proteins having both hydrophilic and hydrophobic regions can be adsorbed at the oil–water interface where they unfold making the system thermodynamically more stable. They can form a film on the interface maintaining the emulsion for long periods. These protein properties depend on protein structure which can be modified by different treatments and is sensitive to environmental conditions such as pH and ionic strength (Turgeon, Gauthier, Mollé, & Léonil, 1992; Pizones Ruíz–Henestrosa, Carrera Sánchez, Pedroche, Millán, & Rodríguez Patino, 2009; Zhang, Jiang, Mu, & Wang, 2009). Enzymatic hydrolysis has been used to change functional properties of proteins (Damodaran & Paraf, 1997; Kim, Park, & Rhee, 1990; Martínez, Carrera Sánchez, Pizones Ruíz–Henestrosa, Rodríguez Patino, & Pilosof (2007); Rodríguez Patino et al., 2007) because it results in the formation of peptides chains of smaller size and a remaining of proteins with different structural characteristics. Many plant protein isolates have been proposed as high quality ingredients presenting different functional properties (Konishi & Yoshimoto, 1989; Marcone & Kakuda, 1999; Van Koningsveld et al., 2002; Palazolo, Sorgentini, & Wagner, 2005; Puppo et al., 2008).

Among them amaranth proteins are an interesting alternative. Amaranth is an ancient crop cultivated in America, which presents many agronomic advantages. Its seeds contain 15–18% protein with a high proportion of lysine and sulfur amino acids. Therefore, the use of amaranth proteins as an ingredient in food formulations is attractive given their high nutritional value (Bressani, 1989;

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doi:10.1016/j.foodhyd.2009.12.008

Marcone, 1999; Martínez, Castellani, & Añón, 1997; Segura–Nieto, Barba de la Rosa & Paredes–López, 1994).

Regarding their functional properties, it has been informed that, similar to albumins, globulins can constitute good emulsifiers (Marcone & Kakuda, 1999; Silva–Sánchez, González–Castañeda, De León–Rodríguez, & Barba De La Rosa, 2004). Emulsifying properties of amaranth isolates are just beginning to be studied. Fidantsi and Doxastakis (2001) have prepared isolates which act as effective emulsion–stabilizing agents, though its foaming properties showed to be better than the emulsion properties. On the other hand Tomoskozi et al. (2008) studied the functional properties of amaranth protein fractions and protein isolates, and found that the emulsifying properties were relatively poor in comparison legume properties. Amaranth proteins showed a low solubility. Taking into account the correlation between solubility and surface properties (Vodjani, 1996); it is possible that structural modifications that increase protein solubility would improve their emulsifying properties. It has been shown that enzymatic hydrolysis improves the solubility of an amaranth protein isolate (Scilingo, Molina Ortiz, Martínez, & Añón, 2002), and it is known that pH exerts great influence on these proteins solubility. On these grounds we tested the possibility of endowing amaranth proteins with good emulsifying properties by means of enzymatic proteolysis. Furthermore, the emulsifying capacity of different amaranth protein hydrolysates and the effect of the pH of the continuous phase of the emulsion were analyzed.

2. Materials and methods

2.1. Amaranth seeds and flour

The seeds of *Amaranthus hypochondriacus* cultivar Artasa 9122 were obtained from INTA, Anguil, La Pampa, Argentina. They were ground in an Udy mill (UDY Corp., USA) 1 mm mesh and screened by 10 xx mesh (Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, Argentina), to obtain the amaranth flour. The latter was defatted by extraction with hexane (1:10 w v^{-1}) at room temperature during 24 h, under continuous stirring during the first 5 h. After drying at room temperature, the flour was stored at 4 °C until used. Protein content of the flour was 23.4 ± 0.8% (w w^{-1}, wb), as determined by the Kjeldahl method (AOAC, 1984) using a factor of 5.85 (Becker, 1981; Scilingo et al., 2002).

2.2. Preparation of amaranth protein isolate (AI)

The amaranth isolate was prepared according to Martínez and Añón (1996). The defatted flour was suspended in water in a 1:10 w v and the suspension was adjusted to pH 9.0 by adding 2 M NaOH. The suspension was stirred during 1 h and then centrifuged at 9 000 g for 20 min at 10 °C. The supernatant was adjusted to pH 5.0 with 2 N HCl and centrifuged at 4 °C for 20 min at 9000 g. The pellet was suspended in a small volume of water, neutralized with 0.1 M NaOH, and freeze–dried. The protein content of the isolate was 73.9 ± 1.4% (wb) as determined by the Kjeldahl method (N × 5.85).

2.3. Preparation of protein hydrolysates

The hydrolysates were prepared by treating the AI either with alcalase (Protease from *Bacillus licheniformis*; Sigma, cat. #P4860) in a concentration of 0.8 μL/g isolate, or with trypsin (Trypsin; Sigma, cat. #T1426, 10 600 BAEE units mg solid) added at 10 mg g^{-1} isolate. The amaranth protein isolate (AI) was dispersed to a final concentration of 10 mg mL^{-1} in 35 mM phosphate buffer (pH 7.8) and was stirred for 1 h at 37 °C, before adding the enzymes. Hydrolysis was carried out for 20 min when the enzyme was trypsin, and for 20 min or 240 min when alcalase was employed. The hydrolysis was stopped by heating at 85 °C in a hot water bath during 10 min. Then the slurry was cooled in an ice bath, frozen, lyophilized and stored at 4 °C until used. The degree of hydrolysis (DH%) was analyzed by the trinitrobenzenesulfonic acid method (TNBS–Adler–Nissen, 1979). The trypsin hydrolysate showed a DH% = 2.2, and was named TH2.2. Hydrolysates obtained by alcalase treatment during 20 min and 240 min showed DH% = 1.7 and a DH% = 9.5, respectively, and were named AH1.7 and AH9.5.

2.4. Preparation of o/w emulsions

The following buffer solutions were used to make the emulsions: 35 mM phosphate buffer pH 2.0, 31 mM NaCl; 35 mM phosphate buffer pH 6.3 and 35 mM tris buffer pH 8.0, 27 mM NaCl. All the solutions had the same ionic strength ($\mu = 0.047$).

The samples (1 mg protein ml) were dispersed in the buffers and stirred for 1 h at room temperature. Emulsions were prepared by homogenizing 4 mL of refined sunflower oil and 16 mL of the protein suspension (20% oil v v) with an ultrasound homogenizer (SONICS Vibra Cell VCX750) at a power level of 50%, making 2 pulses of 5 s each, with the standard tip immersed 1/3 in a glass of 28 mm diameter. At the same time it was mild stirred with a magnetic stirrer to allow the entire mixture pass through the emulsification zone. The temperature of the preparation did not exceed 29 °C during the homogenization process.

2.5. Measurements of oil droplets size

The particle size distribution, mean Sauter diameters ($d_{3,2}$) and mean De Brouker diameters ($d_{4,3}$) of the emulsion droplets were determined using a Coulter LS 230 (Coulter Electronics, USA) laser light scattering instrument. Immediately after making the emulsions a sample was taken and dispersed in recirculation water until it reaches an obscuration level of 8–11% detected by the device measuring cell. To measure the size distribution avoiding flocculation 0.5 mL of the emulsions were poured in a tube containing 3 mL of 1% SDS solution and measured after 1 h of still storage.

The specific interfacial area (SIA) was calculated from the mean $d_{3,2}$ value of the emulsions containing SDS with the equation:

$$\mathrm{SIA} = 6 \times \text{oil ratio}/d_{3,2}$$

Polydispersity (Pd) of the emulsion was calculated from the information provided by the droplet size distribution as follows:

$$\mathrm{Pd} = (d_{0.1} - d_{0.9})/d_{0.5}$$

where 10, 50, 90 percent of the oil volume in the emulsion is contained in droplets with diameters below or equal to $d_{0.1}$, $d_{0.5}$ and $d_{0.9}$ respectively.

2.6. Dynamic light scattering measurements

The stability of the emulsions was determined through the use of a vertical scan analyzer Quick Scan (Beckman–Coulter inc., USA). The samples were loaded into a cylindrical glass measurement cell, and the backscattering percentage profiles (%BS) all along the tube were immediately monitored every 3 min for 3 h as a function of the sample height (total height, 60 mm approximately). Then cells were still stored for 24 h at room temperature and another individual %BS measurement was done. These measurements were used to plot the kinetics of the mean %BS in the lower part of the tube (10–15 mm height) and the upper part of the tube (40–50 mm

height). The $k_{0,1}$ value and the cream destabilization percentage (%CD) were also calculated as follows:

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

based on the mean values of %BS in the lower part of the tube (10–15 mm height), where $t_{0.1}$ is the time of diminish 10% the $\%BS_{in}$ (initial value of %BS). This parameter indicates the stability of the emulsion respect of the creaming process. The increase of $k_{0.1}$ suggests a decrease of the emulsion stability.

$$\%CD = 100 \times (\%BS_{max} - \%BS_{24h})/\%BS_{max}$$

based on the mean values of %BS in the upper part of the tube (40–50 mm height), where $\%BS_{max}$ is the maximum value of %BS and $\%BS_{24h}$ is the value of %BS at the 24 h after the first measure.

2.7. Gel filtration chromatography (FPLC)

The samples AI, AH1.7, AH9.5 and TH2.2 were suspended in the same buffer solutions used for making the emulsions and gently stirred for 1 h, centrifuged for 20 min at 15 000 g 20 °C and filtered through a 0.22 μm membrane filter. The protein concentration was approximately 3 mg mL^{-1}. Five hundred μl of the samples were injected in a Superose 6B HR 10/30 column and analyzed by means of a Pharmacia LKB, FPLC system. The elution was performed with the buffers at a flow rate of 0.2 mL min^{-1}; and the elution profile (absorbance at 280 nm) was obtained. The column was calibrated with blue dextran (V0), thyroglobulin (669 kDa), ferritin (440 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), cytochrome-C (12.4 kDa) and aprotinin (6.5 kDa). The protein fractions identification was performed according to previous data obtained in our lab (Martínez et al., 1997, Scilingo et al., 2002).

2.8. Solubility

Samples AI, AH1.7, AH9.5 and TH2.2 were dispersed in buffers of different pH at 1 mg of protein/ml. Dispersions were mild stirred with a magnetic stirrer for 1 h at room temperature and centrifuged at 15 000 g during 15 min at 20 °C. Immediately it was carried out the solubility analysis using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

3. Results and discussion

3.1. Emulsifying activity

Fig. 1 shows particle size distribution in the absence of SDS, expressed as volume% for the emulsions initially obtained with AI, AH1.7, AH9.5 y TH2.2 at different pHs of the continuous phase (2.0, 6.3, and 8.0). In every case particle size distributions were bimodal, with two definite populations: (I) drops with a diameter ≤ 2 μm, and (II) drops with a mean diameter higher than 10 μm. The contribution of the population I to the total volume of the disperse phase was not significant, but its importance increases if the area created during the homogenization process is considered (insert in Fig. 1a). The distributions for the emulsions obtained with AI exhibit a small change as the pH of the continuous phase varies: for population II, particle size ranges from 10 to 100 μm at pH 2.0,

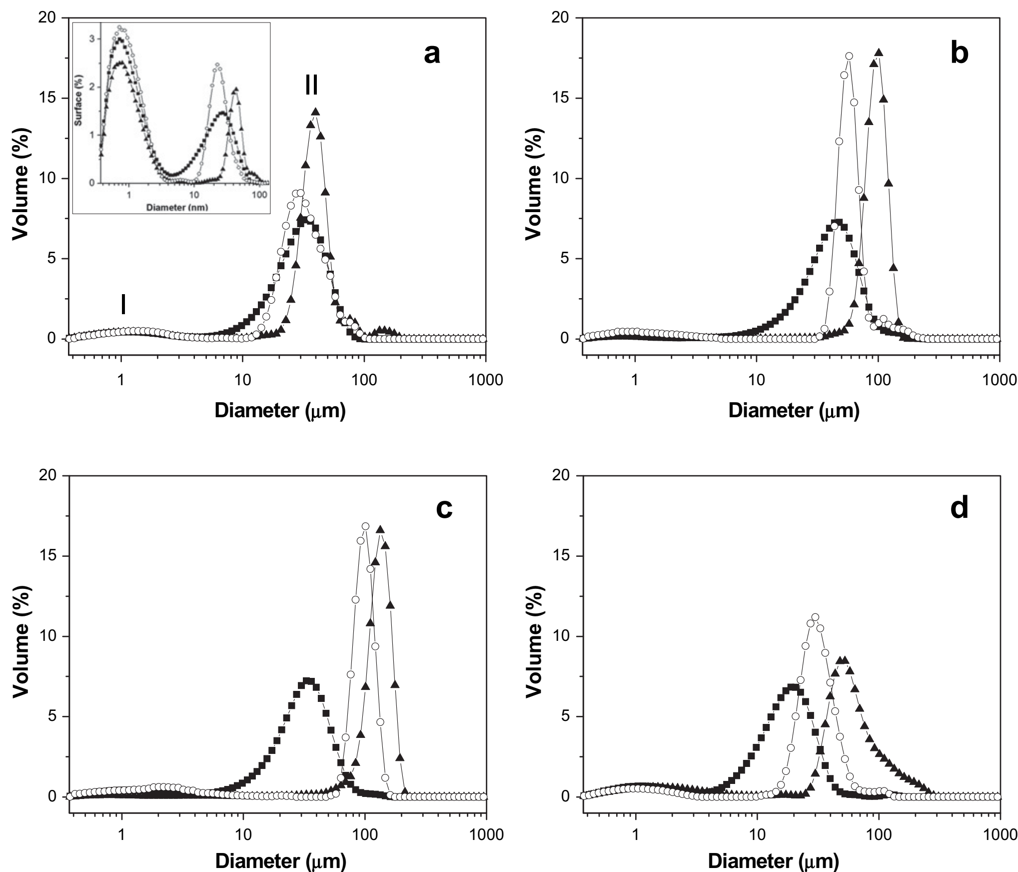

Fig. 1. Profiles of particle size distributions of o/w emulsions prepared with dispersions of non-hydrolyzed, and hydrolysates of amaranth proteins: AI (a) AH1.7 (b), AH9.5 (c), TH2.2 (d) at: pH 2.0 (■), pH 6.3 (▲) and pH 8.0 (○). Distributions were expressed in percentage of oil volume.

which is a wider range than those observed at pH 8.0 and 6.3 (Fig. 1a). For emulsions prepared with AH1.7, AH9.5 and TH2.2, drop size distributions changed significantly with the pH of the continuous phase (Fig. 1b–d). Drop size of population II increased markedly at pH 6.3 and to a lesser extent at pH 8.0, with no important changes detected at pH 2.0 as compared to distributions obtained for AI. At pHs 6.3 and 8.0 the increase in drop size for population II was more evident for emulsions prepared from AH1.7 and AH9.5, this effect being higher for higher hydrolysis degrees (Fig. 1b–c).

Particle size distributions in the presence of SDS showed the same trends discussed previously (Table 1). However, the particle size distributions at pH 2.0 presented lower value drop diameters (<10 μm) than in absence of SDS.

The most polydispersed emulsions were those obtained at pH 2.0 regardless of their formulation from the isolate or from any of the hydrolysates under study (Table 1). A marked reduction of polydispersity was detected for emulsions prepared with AH1.7 and AH9.5 at pH 6.3, which was also observed for AH1.7 at pH 8.0. A reduction of polydispersity was also observed for emulsions prepared from TH2.2 with increasing pH values of the continuous phase.

The specific interfacial area (SIA) by AI vary significantly with pH changes in the continuous phase of the emulsion, with values higher for pH 2.0 (Table 1). In the case of emulsions prepared from hydrolysates, the highest SIA was obtained with hydrolysates at pH 2.0. At pH 8.0 and 6.3 the SIA was 50% and 80% lower than that obtained at pH 2.0 respectively. The decrease of SIA with pH for the AI was lower than that corresponding to hydrolysates.

Initial backscattering (%BS_{in}) profiles obtained as a function of the length of the testing tube provide some information regarding the microstructure of the original emulsion. While %BS_{in} values depend not only on the size of the drop but also on the volumetric fraction of the disperse phase, in the case of recently prepared emulsions the drops are uniformly distributed along the testing tube. In this situation, %BS_{in} values enable a qualitative determination of the drop size of the emulsion. As shown in Fig. 2, initial %BS tends to decrease with increasing $d_{4,3}$ values. Drops of smaller size for all samples with exception of AH1.7 correspond to emulsions at pH 2.0, which reflect a greater proportion of incident light. Similar relations were obtained by Márquez, Palazolo, and Wagner (2005) and Palazolo (2006) for emulsions made of soy milk–vegetal oil and dairy fat, and emulsions made of soy protein isolates and native and denatured soy proteins, respectively.

The comparison of the particle size distributions at pHs 6.3 and 8.0 for the different hydrolysates obtained indicates that the action of alcalase led to the peptides with less emulsifying activity - large particle sizes and small SIA - trend that intensified with the increasing degree of hydrolysis.

Table 1
Initial parameters of the emulsions prepared with protein samples at different pHs. Maximum standard deviation was: $d_{4,3}$: 4%, $d_{3,2}$ and SIA: 6%.

Sample	$D_{4,3\ av}$ (μm)	Polydispersity	$D_{3,2\ av}$ $(\mu m)_{SDS}$	$D_{4,3\ av}$ $(\mu m)_{SDS}$	SIA $(m^2/ml_{em})_{SDS}$
AI pH2	31.1	1.4	4.2	14.7	0.28
AI pH 6.3	46.4	0.9	9.8	–	0.12
AI pH 8	30.1	1.2	7.9	26.3	0.15
AH1.7 pH 2	44.7	1.6	3.6	10.0	0.33
AH1.7 pH 6.3	94.6	0.5	22.5	86.6	0.05
AH1.7 pH 8	53.4	0.6	6.9	38.9	0.17
AH9.5 pH 2	28.9	1.6	3.2	7.4	0.38
AH9.5 pH 6.3	131.0	0.6	24.6	116.3	0.05
AH9.5 pH 8	82.8	1.2	15.3	87.2	0.08
TH2.2 pH 2	18.5	1.7	3.4	8.7	0.35
TH2.2 pH 6.3	67.8	1.5	13.5	71.0	0.09
TH2.2 pH 8	32.3	1.0	9.1	37.4	0.13

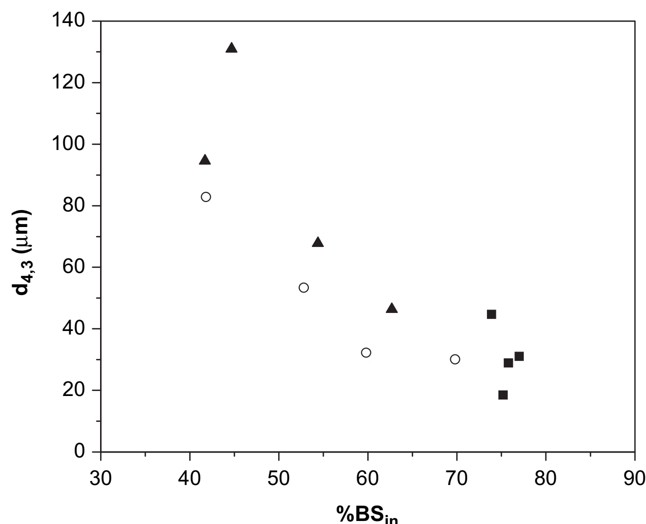

Fig. 2. Relationship between $d_{4,3}$ and %BS_{in} of o/w emulsions prepared with dispersions of non-hydrolyzed, and hydrolysates of amaranth proteins: AI, AH1.7, AH9.5 and TH2.2 at: pH 2.0 (■), pH 6.3 (▲), pH 8.0 (○). Maximum standard deviation was: $d_{4,3}$: 4%, %BS_{in}: 3%.

3.2. Emulsifying stability

To evaluate the creaming–flocculation kinetics of the emulsions under study, the %BS profiles during the first 3 h and at 24 h of still storage were analyzed.

The migration of drops in an oil: water emulsion proceeds to the upper part of the measuring tube, thus leading to a diminution of drops in the lower portion of the tube and a reduction of %BS. Therefore, creaming–flocculation kinetics were determined from mean %BS values (%BS_{av}) as a function of time in the zone of the tube ranging from 10 to 15 mm. Fig. 3a shows the creaming–flocculation kinetics corresponding to emulsions made from AI at the three different pH values chosen. Emulsions prepared at pH 6.3 exhibited the highest degree of destabilization due to a rapid drop of %BS_{av} with time, followed by emulsions made at pH 8.0. Emulsions prepared at pH 2.0 were stable for longer than 30 min. In addition, in the later case the clarification of the lower aqueous phase at 40 min of storage was significantly lower than that of emulsions made at pH 6.3 and 8.0. Similar changes with the pH of the continuous phase were observed for emulsions prepared from AH1.7, AH9.5 and TH2.2. Emulsions obtained with AH1.7 and TH2.2 exhibited a higher stability, particularly at pH 2.0. The behavior of the emulsions after 24 h (1440 min) of still storage follows the same trend that one observed during the first hours. Fig. 4 shows the destabilization constant $k_{0.1}$ which provides data on the clarification rate of the lower portion of the measuring tube (10–15 mm) until yielding a 10% reduction of the initial BS value. It can be observed that the most unstable emulsions are those prepared with hydrolysates obtained by alcalase treatment at pHs 6.3 and 8.0.

Flocculation–coalescence kinetics of the cream phase were determined from the variation of the %BS_{av} in the upper portion (40–50 mm) of the measuring tube. The results obtained for all the assayed samples are shown in Fig. 5. For emulsions prepared from AI at pH 6.3 and 8.0, an initial reduction of %BS_{av} can be observed, followed by an increase of this parameter until reaching a virtually constant value at 28 min (pH 6.3) or 40 min (pH 8.0).

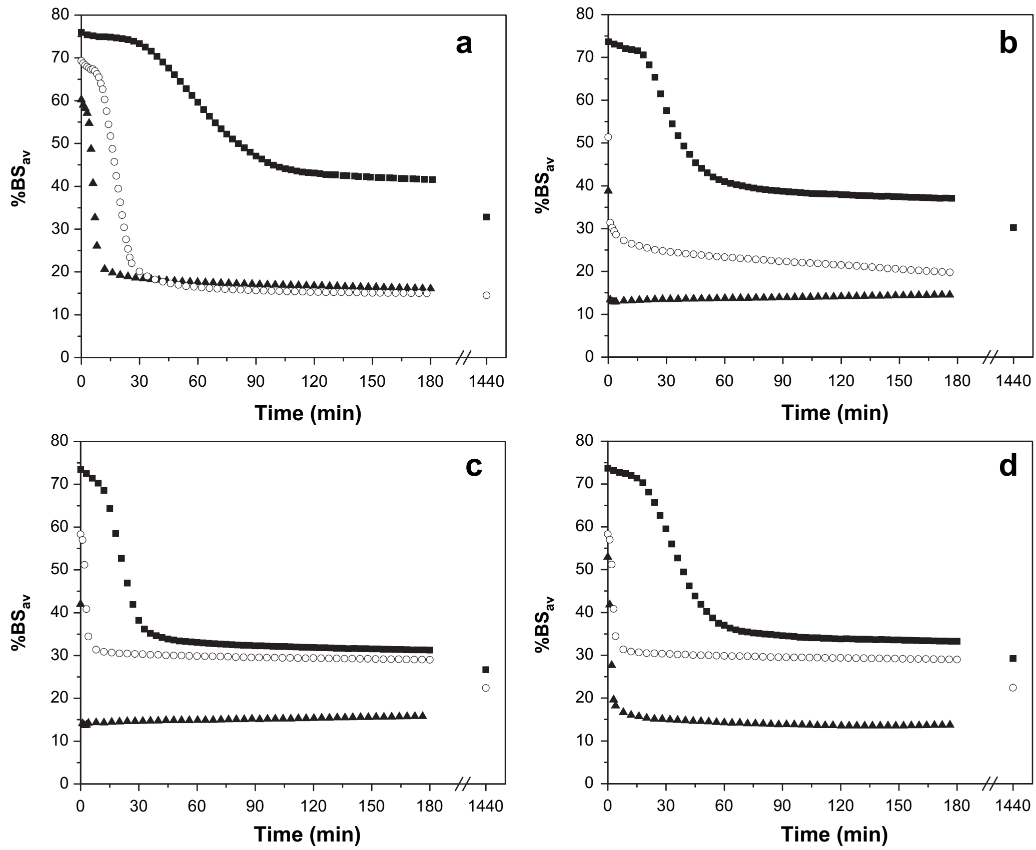

Fig. 3. Destabilization kinetics, clarification, Zone 10–15 mm, corresponding to o/w emulsions prepared with dispersions of AI (a), AH1.7 (b), AH9.5 (c) and TH2.2 (d) at: pH 2.0 (■), pH 6.3 (▲) and pH 8.0 (○). Values at 1440 min (24 h) that do not appear in the figure had a %BS$_{av}$ lower than 5%.

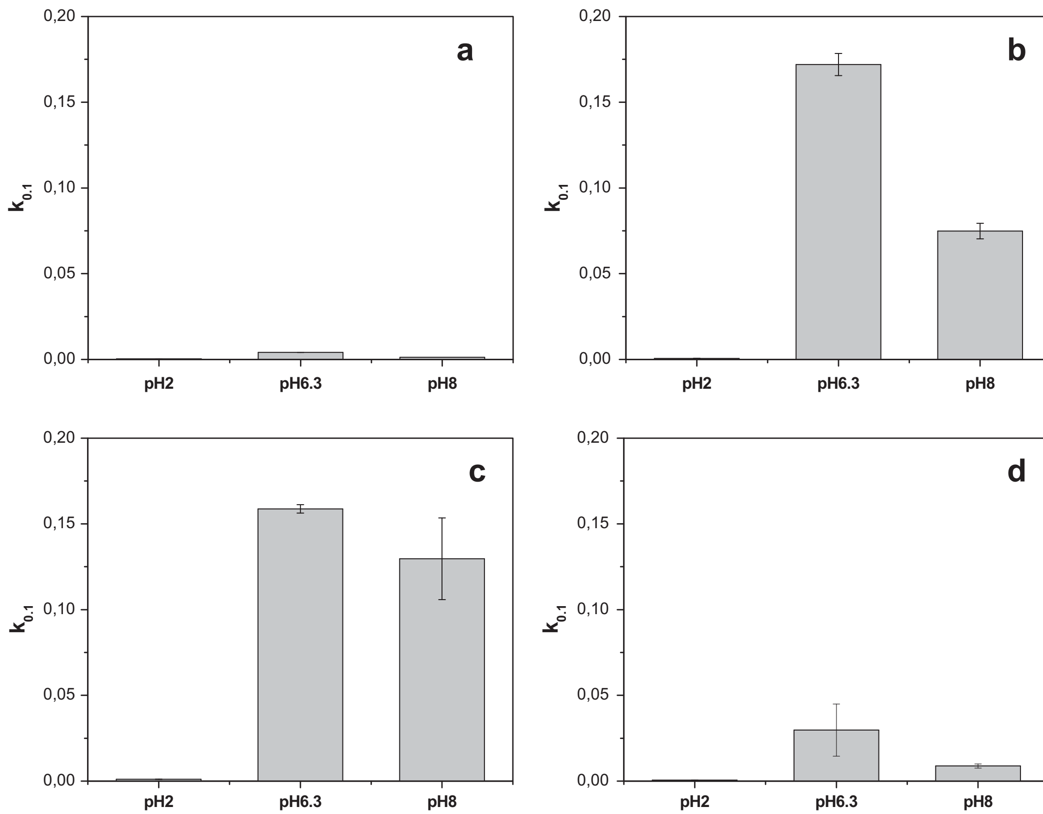

Fig. 4. Constant of creaming, $k_{0.1}$, at different pHs corresponding to o/w emulsion prepared with dispersion of AI (a), AH1.7 (b), AH9.5 (c) and TH2.2 (d).

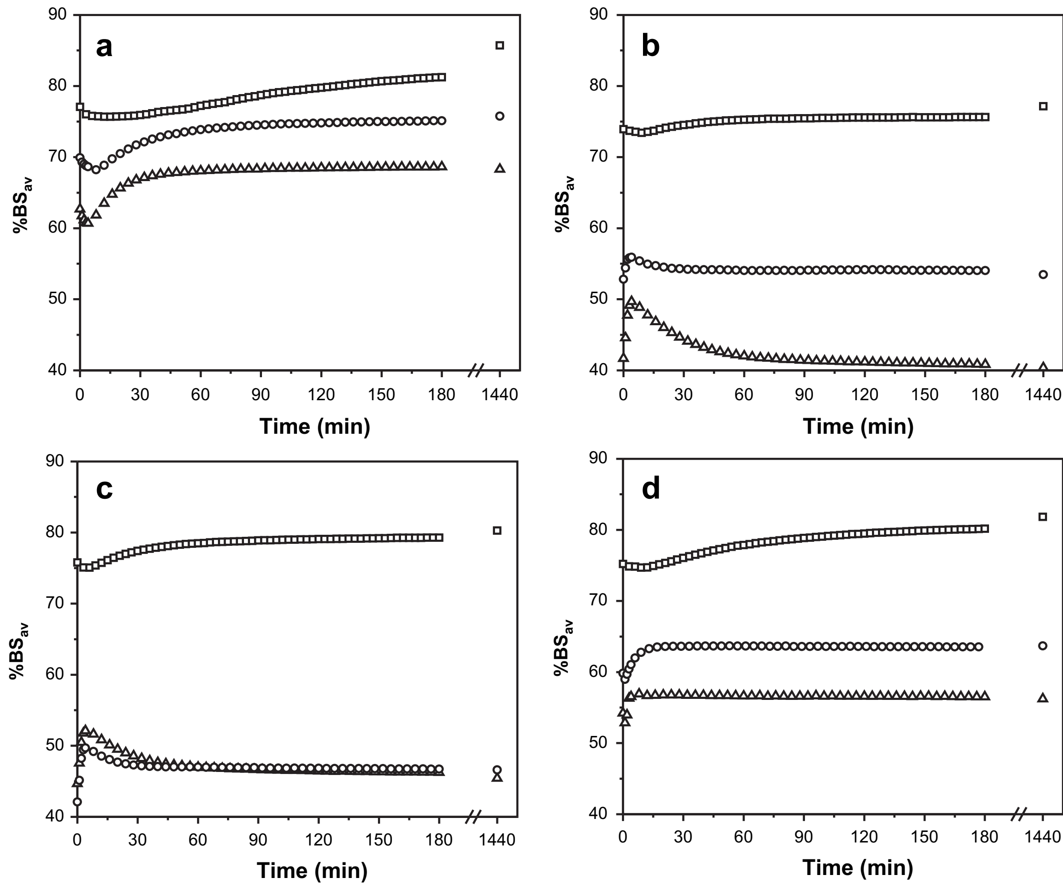

Fig. 5. Destabilization kinetics, coalescence of the cream phase, Zone 40–50 mm, corresponding to o/w emulsions prepared with dispersions of AI (a), AH1.7 (b), AH9.5 (c) and TH2.2 (d) at: pH 2.0 (■), pH 6.3 (▲) and pH 8.0 (○).

This behavior of the $\%BS_{av}$ is due to the variation in the number and size of the drops present in the cream phase. The ascent of drops from the lower phase is accompanied by an increase in $\%BS_{av}$, which occurred more rapidly for the emulsions made at pH 6.3, in agreement with the data depicted in Figs. 3 and 4. The emulsions prepared at pH 2.0 exhibited only a slight $\%BS_{av}$ increase with time, evidencing its higher stability in the flocculation–creaming process. The behavior of emulsions prepared with AH1.7 and AH9.5 was markedly different at pH 6.3 and 8.0, since both emulsions showed a reduction of $\%BS_{av}$ with storage time, indicating a coalescence process that predominates over the increase of drops in the cream phase while in AI and TH2.2 it could be seen only a little coalescence at pH 6.3 within 24 h of storage. The behavior of emulsions prepared from TH2.2 was similar to that of emulsions made from AI, but $\%BS_{av}$ values were slightly lower (especially at pHs 6.3 and 8.0). Fig. 6 depicts the destabilization percentage of the cream phase (%CD) considering the behavior of the emulsions at 24 h of still storage. These results confirmed the tendency observed for the behavior of the emulsions during the first 3 h after preparation. The highest destabilization percentages of the cream phase corresponded to AH1.7 and AH9.5 emulsions at pHs 6.3 and 8.0. A clear diminution of the light dispersed by particles was observed in these cases, which was a consequence of the reduction in the number of particles and increase of their size due to the coalescence phenomena detected.

The destabilization of emulsions by coalescence previously discussed was verified by optical microscopy of the emulsions formed recently and after 3 and 24 h of still storage. The results showed the same trend of those obtained by Quick Scan.

De Brouker diameters ($d_{4,3}$) in the presence or absence of SDS corresponding to the emulsions initially obtained are shown in Table 1. For all emulsions at pH 2.0, $d_{4,3}$ exhibited a reduction of mean values in the presence of SDS, suggesting the formation of floccules between particles. However the mean diameter of floccules at pH 2.0 was lower or equal than size of the individual droplets of emulsions prepared at pH 6.3 and 8.0.

The analysis of the results at pHs 6.3 and 8.0 of the continuous phase clearly shows that the hydrolysis of amaranth proteins with alcalase significantly reduces its action as a stabilizer in oil - water emulsions especially against flocculation-coalescence.

3.3. *Relationship between emulsifying capability and physicochemical characteristics of proteins*

Structural characteristics of proteins and polypeptides, especially size, charge, molecular flexibility and surface hydrophobicity, influence on their ability to form and/or stabilize emulsions. Treatments applied to amaranth proteins in this study, together with the pH variation in the continuous phase of the emulsions, affected the solubility, the molecular size and the folding degree of amaranth proteins. The low grade hydrolysis used in the current study affected the solubility of amaranth proteins and polypeptides at the pH values used for the continuous phase of the emulsion. The highest solubility of the samples (63–82%) has obtained at pH 2.0, followed by that obtained at pH 8.0 (48–66%), and finally by that measured at pH 6.3, at which solubility values not only were the lowest but also exhibited the widest range (24–59%) (Table 2). It is important to point out that the isoelectric point of proteins present

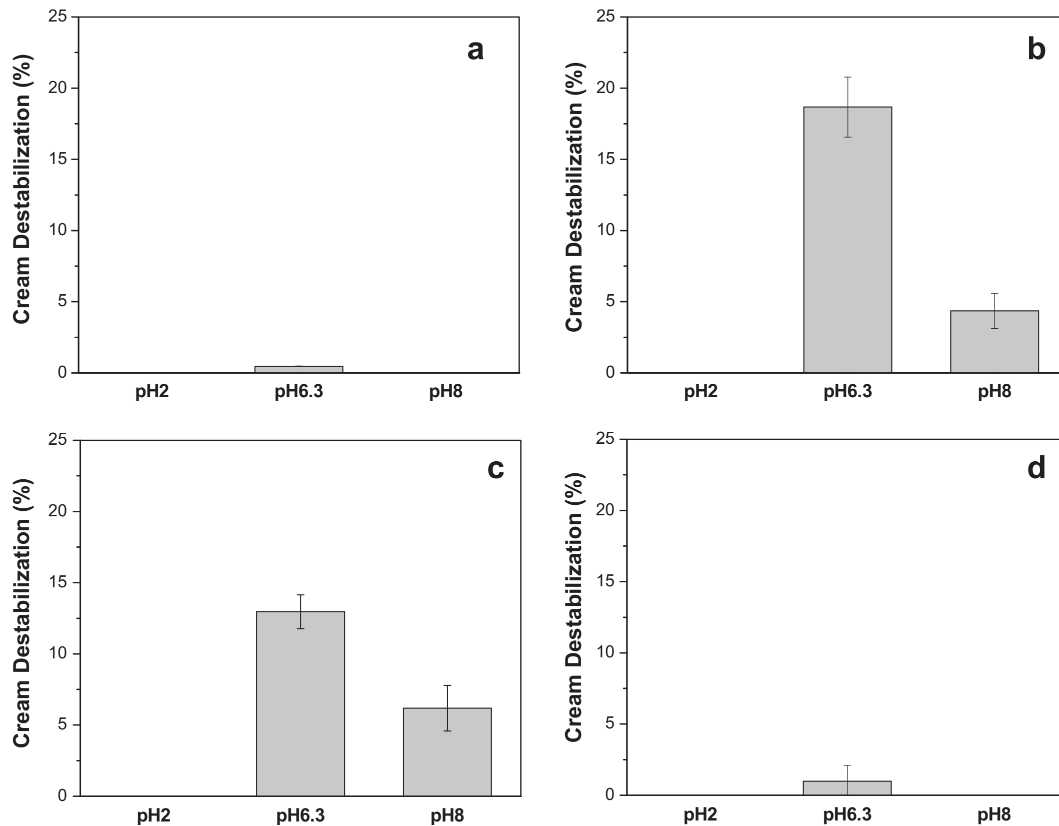

Fig. 6. Cream destabilization at different pHs corresponding to o/w emulsion prepared with dispersion of AI (a), AH1.7 (b), AH9.5 (c), TH2.2 (d).

in the amaranth isolate is between 4.5 and 6.0 (Marcone & Yada, 1992). In every case, the samples showing the highest solubility were those prepared from AI and those with the lowest solubility were those obtained from AH1.7. Previous results obtained in our laboratory indicate that at degree of hydrolysis equal to or above 16% the solubility of the alcalase hydrolysates was higher - in all the pH range – than that corresponding to the AI. (Vecchi, 2007).

Trypsin and alcalase are members of a serine protease family. The first one cleaves the peptide bond on the carboxyl side of arginine and lysine (Kishimura et al., 2007) while the second hydrolyzes peptide bonds with broad specificity liberating peptides with hydrophobic amino acids such as Phe, Tyr, Trp, Leu, Ile, Val, Met at their C-terminal. Condés, Scilingo, and Añón (2009) showed that amaranth 11S and globulin P were the main targets for the trypsin, while a polypeptide of 45 kDa from 7S globulin was more resistant to trypsin action. Meanwhile Vecchi (2007) showed that alcalase hydrolyzed rapidly polypeptides of molecular weight higher than 30 kDa present in amaranth. The B polypeptides of 11S globulin, hydrophobic in nature, presented the greater resistance to alcalase hydrolysis. According to Chabanon, Chevalot, Framboisier, Chenu, and Marc (2007) the hydrolysis of rapeseed proteins with alcalse seemed to be carried out over the whole proteins also leading to the appearance of high proportion of intermediary peptides following a zipper mechanism.

According to the solubility results obtained and taking into account the mechanism of action of alcalase is possible that during the early stages of the hydrolysis, this enzyme release hydrophobic peptides able to interact and form insoluble aggregates stabilized by hydrophobic interactions. This phenomenon would be favored at pHs closer to the average PI of these proteins.

As revealed by size exclusion chromatography (Fig. 7), soluble fractions differed in the distribution of molecular sizes. The chromatography profile showed the presence of globulin-P polymers (component I, involving a range of elution volumes with MM higher than 500 kDa), globulin-P and 11S-globulin molecules of 280 ± 7 kDa (component II), which were described elsewhere (Martínez et al., 1997) and species of MM < 100 kDa (component III, involving a range of elution volumes mainly composed by albumins). At pH 8.0 the soluble fraction of AI exhibited about 18% of high molecular mass aggregates and hexameric and trimeric forms of 11S and 7S, about 6.5% of monomers, and the remaining components were low molecular mass proteins and polypeptides. The soluble fractions of alcalase hydrolysates did not contain high molecular mass aggregates, but contained a higher proportion of monomeric species and low molecular mass polypeptides resulting from hydrolysis. Trypsin hydrolysates contained not only these components, but also soluble aggregates, albeit in a much lower proportion than that found in the AI and exhibiting an intermediate molecular mass (corresponding to molecular masses from 68 to 14 kDa). Species found in the soluble fraction at pH 6.3 were equivalent to those detected at pH 8.0, while at pH 2.0 only polypeptide species of low molecular mass were observed, even in the case of the non-hydrolyzed isolate, thus implying the existence of

Table 2
Solubility (%) of protein samples at different pHs.

	pH 2.0	pH 6.3	pH 8.0
AI	82.2 ± 1.8	28.4 ± 1.2	63.6 ± 2.0
AH1.7	63.4 ± 2.1	23.7 ± 1.5	49.1 ± 1.8
AH9.5	70.2 ± 1.2	36.1 ± 1.0	57.1 ± 1.0
TH2.2	76.9 ± 3.0	59.1 ± 3.0	66.4 ± 1.8

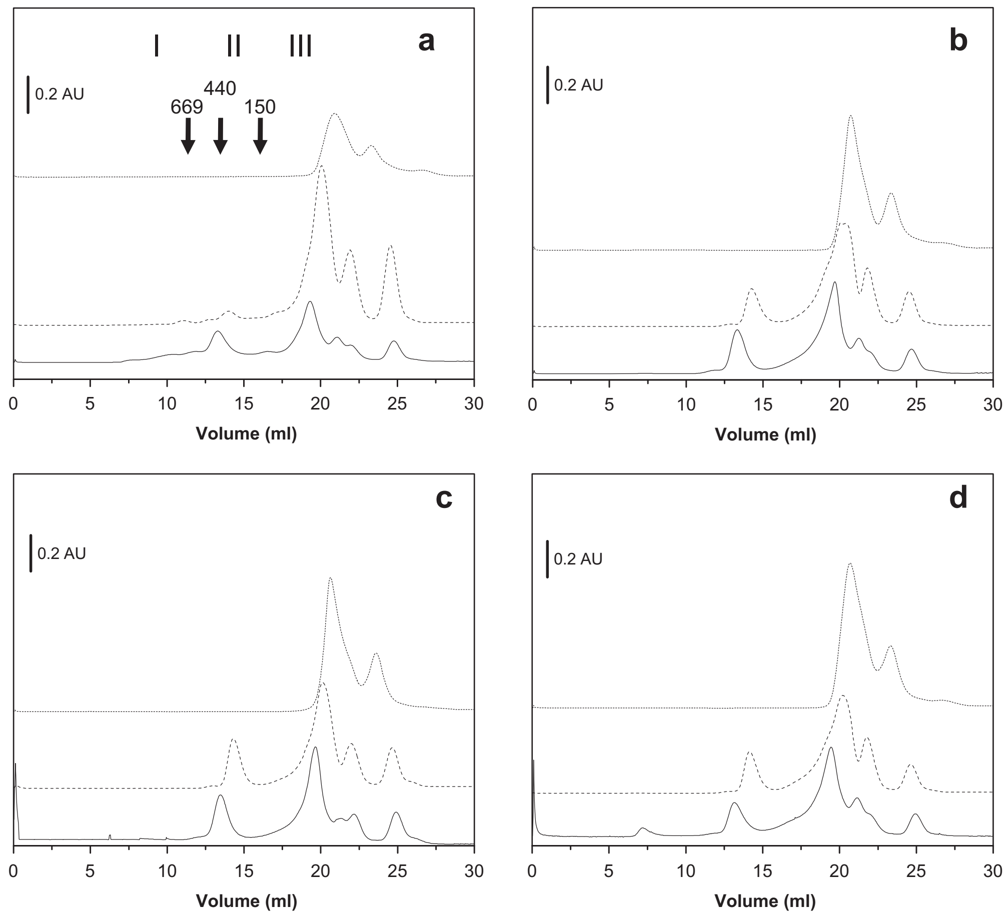

Fig. 7. Exclusion chromatography (FPLC) of protein dispersions of AI (a), AH1.7 (b), AH9.5 (c), TH2.2 (d) at: pH 2.0 (dotted line), pH 6.3 (dashed line), pH 8.0 (drawn line). Arrows indicate elution volumes of standard proteins (molecular masses in kDa).

dissociation reactions and of aggregates of very high molecular mass not detected in this assay.

It must be noted that amaranth proteins and polypeptides are completely denatured at pH 2.0, at variance with what happens at pH 6.3 and 8.0 according to data obtained by DSC (results not shown).

The above described results clearly show that AI contains proteins species capable of forming and stabilizing emulsions, mainly at acidic pH (2.0) and to a lesser extent at pH 8.0. While the emulsions obtained are sensitive to creaming and flocculation, they do not undergo destabilization by coalescence, only a little at pH 6.3. The emulsions prepared from proteins subjected to low grade trypsin hydrolysis (TH2.2) are also sensitive to creaming–flocculation, whereas alcalase–hydrolyzed proteins (AH1.7 and AH9.5) exhibited a significant destabilization by creaming, flocculation and coalescence, at pHs 6.3 and 8.0.

It is known that the creaming rate of a spherical particle is primarily dictated by the Stokes law, although the latter has several limitations for describing the behavior of emulsions, including the existence of Brownian motion, the intrinsic polydispersity of emulsions, the colloidal interactions between particles, the hydration degree of the drops, and the rheological behavior of the continuous phase (McClements, 1999). The emulsions obtained at pH 2.0 prepared with non-hydrolyzed amaranth proteins showed the highest degree of flocculation and the lower creaming rate. In this conditions the polypeptides present a net positive charge and are unfolded exposing the hydrophobic residues promoting flocculate formation by hydrophobic interaction. Otherwise, the higher creaming stability of emulsions obtained at pH 2.0 could be attributed to the higher polydispersity produced under these conditions, and also to the formation of a network opened of floccules that would affect the movements of the drops.

The fact that the smaller drops and the absence of coalescence are found in emulsion prepared at pH 2.0, which have a predominance of low molecular weight polypeptides resulting from dissociation and/or hydrolysis of amaranth proteins, would indicate that these proteins improve the emulsifying activity and are able to form a resistant film at the interface. According to assays currently in course in our laboratory, isolates and hydrolysates of amaranth form films with very good rheological characteristics at pH 2.0 (not shown). These results are consistent with those reported by Martin, Bos, and van Vliet (2002) with soybean proteins. This authors found that both 3S (form, predominant at acidic pH) and 11S glycinin may form cross linkage but 3S presents an easier and higher rate of unfolding and rearrangement in the interface due to the higher flexibility resulting from electrostatic repulsion forces within the molecule.

The decreased solubility and the consequent presence of a higher amount of aggregates, together with the existence of smaller protein species due to hydrolysis and the nature of the polypeptides obtained – hydrophobicity and molecular size -, would account for the coalescence phenomena detected in emulsions prepared from AH1.7 and AH9.5. At pH 6.3 the coalescence of emulsions prepared with alcalase–treated isolates was prominent, in agreement with the lower solubility measured at this pH. Notwithstanding, the later parameter would not constitute the only

cause of destabilization through coalescence, since AI coalesces only a little and has a solubility equal or lower than that of AH1.7 and AH9.5. The type and quantity of interactions of molecules at the interface are also of vital importance in the formation of an interfacial viscoelastic film resistant to coalescence.

In the present work the best emulsions were obtained, for all samples assayed, at pH 2.0. In spite of the characteristic of proteins used as tensioactives (hydrolyzed and non-hydrolyzed proteins) the acidic pH modifies the molecule structure in a manner that allows better penetration at the interface and greater interaction between them favoring the stability of the emulsions obtained. At pH 2.0 the hydrolysates showed a slightly larger emulsifying activity. The effect of the hydrolysis degree is more important at pH 6.3 and 8.0, being less stable the emulsions obtained with alcalase hydrolysates.

Acknowledgements

The author acknowledges the financial support from the Agencia Nacional de Promoción Científica y Tecnológica (FONCyT, PICT 04–12085), European Union (project contract number 032263) the Universidad Nacional de La Plata, Argentina (Project 11/X379).

Authors wish to thank to Laboratorio de Asfaltos, YPF, Refinería La Plata, Provincia de Buenos Aires for kindly providing the Coulter LS–230 particle analyzer.

M. C. Añón is researcher of the of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and J. Ventureira is research fellow of CONICET.

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