Emulsifying Properties of Hydrolysates Isolated from Soybean Protein

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Abstract: We have developed new surfactant agents based on hydrolyzed soybean proteins using papain, and we have studied their ability to form and stabilize emulsions. The interfacial behavior and the emulsifying properties were correlated to the structural changes that the proteins underwent. The hydrolysis reaction was stopped by dropping to pH 2 in one case, or rapidly dropping the temperature to -18°C in the other. The structural and functional properties of the obtained products depended on the way the papain hydrolysis of the soy proteins was stopped. Hydrolysis did not have a beneficial effect on the emulsifying properties of those hydrolysates that were stopped by freezing. For all the degrees of hydrolysis we studied, the emulsifying properties of the isolates were significantly improved when the hydrolysis reaction was stopped by dropping to pH 2.

Keywords: Soy Protein, Enzymatic Hydrolysis, Emulsion Properties

1. Introduction

Soy proteins in numerous and diverse food products. They have a great potential to substitute meat and dairy products considering their high digestibility and their well-balanced amino-acid composition. Nevertheless, over 95% of it is used as animal feed [1]. One of the main uses in human foodstuffs is as a functional ingredient, owing to its ability to form and stabilize gels, emulsions and foams [1]. Despite its commercial importance, the basic knowledge on the emulsifying properties of soy proteins has been scarce compared to their gelifying properties [2].

A restrained proteolysis may have positive effects on the surface activity of these proteins and hence on their emulsifying properties [3]. Studies on the modification of soy proteins by papain hydrolysis and its consequences on structural changes and functional properties of the modified proteins have been made in the past [4],[5]. However, in recent years there has been great progress in the methods and techniques for analyzing the formation and stability of emulsions, allowing for new studies and approaches that help to elucidate how the functional surface properties are linked to the structural characteristics of proteins. Furthermore, more research is needed to improve the functional properties of proteins, especially soy proteins, at pH ranges above than 4.0, so that the use of these proteins in food systems becomes more widespread[1].

In that regard, our goal was to study the emulsion-forming and emulsion-stabilizing properties of the obtained hydrolyzed products, and establish how the interfacial behavior and emulsifying properties of these new surfactant agents relate to the way the hydrolysis reaction is interrupted.

2. Materialsy Methods

2.1. Preparation of the Soy Protein Isolate

The soy protein isolates (SPIs) were prepared by isoelectric precipitation (pH 4.5) of an aqueous solution (alkaline medium at pH 8.0) of defatted soybean meal, which was then re-dispersed in an alkaline medium (pH 8.0) and freeze-dried [6].

2.2. Preparation of the Hydrolysates

The soy protein isolate (about 30 mg of solid per mL) was incubated with papain enzyme solution (0.2 mg of papain per

mL), at a 4:1 (v/v) ratio, in a thermostatic water bath with continuous stirring at 40ºC. SPIs with protein contents of 90.8 ± 0.4 g/100g, 3.50 ± 0.02 g/100g moisture and 5.7 ± 0.02 g/100g minerals (ash) were used. Papain (from Sigma) contained 28 units/mg (one enzyme unit hydrolyzes 1.0 μmol of α-N-benzoyl-L-arginine ethyl ester (BAEE) per minute at pH 6.2 and 25ºC). The solutions or dispersions of the substrates were prepared in a pH 8.0 buffer (0.01M sodium phosphate). Different times for the hydrolysis treatment were used to obtain different degrees of hydrolysis(0. 1.8 - 2.5 and 6.0 %). The hydrolysis reaction was stopped by: 1) dropping the pH of the soy protein isolate hydrolyzates (SPIH) to pH 2 with 6N hydrochloric acid (HpH), and 2) rapid temperature drop of the SPIH dispersion using acooling bath(ice-sodium chloride bath) at -18ºC (HC). Subsequently, the obtained SPIHs were freeze-dried. SPIHs with the same degree of hydrolysis and stopping method were obtained from SPIs belonging to the same batch.

2.3. Determination of the Degree of Hydrolysis

The degree of hydrolysis (DH) was determined by measuring the free amino groups by the trinitrobencensulphonic acid (TNBS) method [7] with modifications described by [8]. The determinations were done three times using an L-leucine standard curve and measuring the absorbance at 420 nm.

2.4. Preparation of the Emulsions of the Study

25:75 (v/v) oil-in-water (o/w) emulsions were prepared using the different obtained hydrolysates, with 1.0 and 2.0 mg/mL protein concentration, dissolved in pH 7, 0.01M sodium phosphate buffer without NaCl. The emulsions were prepared at room temperature in an Ultra-turrax T25 homogenizer using the S 25N- 10G dispersing element at 20,000 rpm for one minute.

2.5. Determination of the Mean Droplet Size in the Emulsions

The droplet size analysis was made with emulsions prepared as previously described, with or without treatment with pH 8.0, 50 mM Tris/HCl solution containing a 1% SDS solution at a 1:1 ratio[9]. A Malvern Mastersizer Micro particle size analyzer was used to determine the $D_{3.2}$ y $D_{4.3}$ mean droplet diameters in a diameter interval ranging from 0.3 to 300 μm. The difference in the mean diameter values with or without the SDS solution was taken as a measurement of floccule or aggregate formation inside the emulsion (degree of flocculation) [9]:

$$DoF =[(D-D_{SDS})/D_{SDS}]x100 \qquad (1)$$

Where DoF is the degree of flocculation, D is the mean diameter as determined in absence of SDS, and D_{SDS} is the mean diameter as determined in presence of SDS.

The determinations were performed twice.

2.6. Protein Concentration at the Interface

The oil droplets were separated (washed) from the non-adsorbed proteins according to the method described by [10] and modifications by[11].The adsorbed and non-adsorbed protein concentrations were determined with the method used by [12].The protein concentration at the interface was calculated as:

$$\Gamma(mg/m^2) = [P_a] \ (mg/mL \ emulsion)/ \ Sv \ (m^2/mL \ emulsion) \ (2)$$

Where Γ is the protein concentration at the interface, $[P_a]$ is the protein concentration adsorbed in the emulsion and Sv is the interface area.

The determinations were performed twice.

2.7. Analysis of the Ability to form and Stabilize Oil-in-Water Emulsions

All the emulsions were studied with an optical vertical scanner (Turbiscan). The creaming and destabilization kinetics of the emulsions were determined by measuring the backscattering value every minute for a total of 60 minutes, as well as a single measurement, 24 hours later. The creaming stability was studied based on the decrease in the meanbackscattering values in the lower part (10-20 mm, BS_{p10-20} zone) of the measurement tube versus time [13]. The destabilization kinetics was analyzed as suggested by [14] using the following equations:

$$BS(t) = BS_h/ (BS_h k_h t + 1) \qquad (3)$$

$$BS(t) = BS_h/ (BS_h k_h t + 1) + (2^2.BS_s)/[2^2 + (k_s.BS_s)^2.t^2] \ (4)$$

Where BS(t) is the BS value at a time-point "t", k_h is BS variation rate versus time constant for a second order kinetic model, k_s is the BS variation rate versus time constant for a sigmoid-fit kinetic model, and BS_h and BS_s are the amplitude parameters for the aforementioned kinetic models, so thatBS_h + BS_s = BS_i.

The overall destabilization of the emulsions was studied using the meanbackscattering values in the 50 - 53 mm area (BS_{p50-53}) versus time and the calculation of D(%)[15] as follows:

$$D (\%) = [(BS_{pmax.} - BS_{p24})/ \ BS_{pmax}]x100 \qquad (5)$$

Where: D(%): overall degree of destabilization, $BS_{pmax.:}$ mean of the maximumbackscattering value at a time-point in a given interval and $BS_{p24.:}$mean backscatteringat 24 hours in a given interval.

The determinations correspond to duplicates which were also assayed twice.

2.8. Statistical Analysis

The fixed-effects model of analysis of variance (ANOVA) was used for statistical treatment of data, with α = 0.05; followed by the comparison of mean values by the least significant differences (LSD) test, with α = 0.05, using Statgraphics plus 7.0 software.

3. Results and Discussion

3.1. Formation of the Emulsion

3.1.1. Particle Size Analysis

The variation of the $D_{3.2}$ and $D_{4.3}$ values of the emulsions in presence of SDS and relative to the different variables under study is shown in Table 1 A marked difference between the $D_{3.2}$ and $D_{4.3}$ values of all assayed samples can be noted, which would indicate that the emulsions are polydisperse [16]. This can be seen in Figure1, where the amplitude of the particle size interval ranges from about 0.3 to 220 μm. All emulsions showed a modal distribution. Most of the oil volume corresponds to the peaks belonging to the higher droplet-size interval, whose diameter is generally above 10 μm. However, Figures1 b and c show that the diameter of the great majority of particles is below 10 μm, which would mean that about 60 to 80% of the emulsified oil volume is distributed among very few droplets, representing less than 0.05% of the total. Most of the newly-formed area percentage, about 70 to 85%, was shown to correspond to the particle size interval below 10 μm, in line with the particle number distribution.

The degree of hydrolysis did not significantly affect $D_{3.2}$ values (Table1). Emulsions that were prepared by HpH showed a significantly higher $D_{3.2}$ value than the corresponding HC emulsions.

The degree of hydrolysis did not significantly affect the $D_{4.3}$ values (Table1). For those emulsions that were prepared by HC, the values corresponding to DH: 0, 1.8, and 2.5% were significantly lower than that of DH 6%, as shown in Figure 1a, where the distribution of volume for HC-DH 6% shows a peak beyond100 μm, whereas the distribution of HC-DH 0% has already decreased by that particle size. On the other hand, in those emulsions prepared by HpH the effect of the degree of hydrolyison $D_{4.3}$ was not detected. The emulsions prepared by HpH showed a significantly higher $D_{4.3}$ value than their correspondingHC counterparts, except for DH 6% where the opposite was true (Figure 1a).

Table 1. Mean $D_{3.2}$ and $D_{4.3}$ values of the studied emulsions, with and without SDS treatment and DoF ($D_{3.2}$).

SPIH	With SDS		Without SDS		
	$D_{3.2}$	$D_{4.3}$	$D_{3.2}$	$D_{4.3}$	DoF ($D_{3.2}$)
HC-DH 0%	5.2 ± 0.3	23 ± 1	12.6 ± 0.8	44 ± 4	142 ± 8
HC-DH 1.8%	5.2 ± 0.3	24 ± 1	12.1 ± 0.8	51 ± 5	133 ± 8
HC-DH 2.5%	5.2 ± 0.3	24 ± 1	11.9 ± 0.8	54 ± 5	129 ± 7
HC-DH 6.0%	4.9 ± 0.3	61 ± 5	10.2 ± 0.8	83 ± 7	108 ± 7
HpH-DH 0%	11.3 ± 0.4	37 ± 1	11.1 ± 0.4	40 ± 2	NF
HpH-DH 1.8%	12.5 ± 0.4	38 ± 1	12.0 ± 0.4	41 ± 2	NF
HpH-DH 2.5%	10.1 ± 0.4	35 ± 1	12.6 ± 0.4	40 ± 2	25 ± 1
HpH-DH 6.0%	9.9 ± 0.4	37 ± 1	12.6 ± 0.4	41 ± 2	27 ± 1

NF: found no flocculation

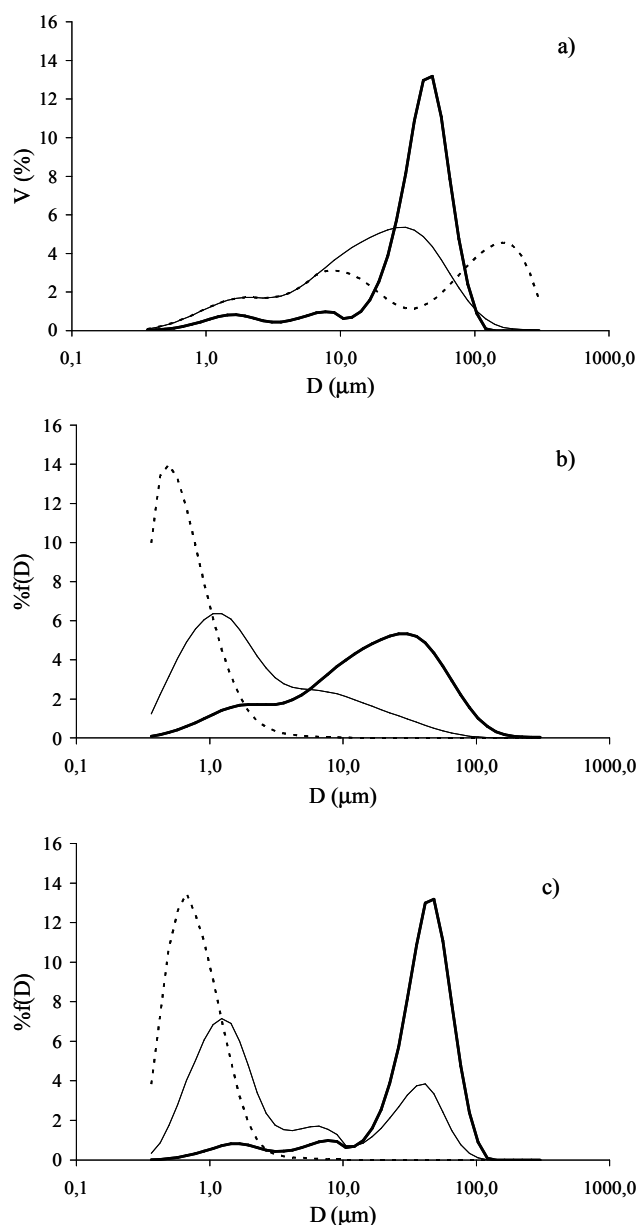**Figure 1.** a) Droplet size distribution of the emulsions at pH 7.0 and [NaCl] = 0 M, with [protein] = 1 mg/mL HpH-DH = 0% (━), HC-DH = 0% (▪▪▪) and HC-DH = 6% (…). The measurements were done in presence of SDS.b) Droplet size distributions of the emulsions at pH 7.0 with HC-DH = 0% containing SDS, c) Droplet size distributions of the emulsions at pH 7.0 with HpH-DH =0%, with SDS. Volume (━), area (−) and number (…).
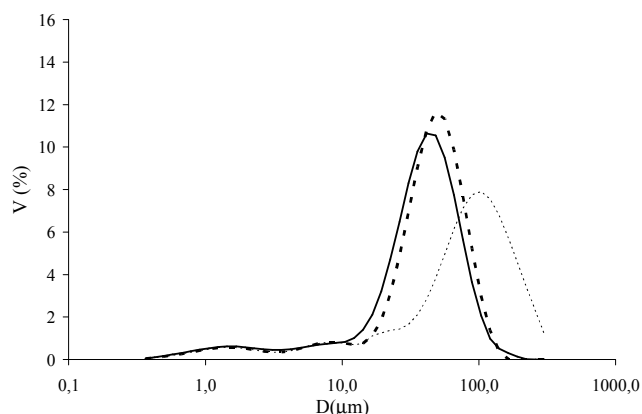
3.1.2. Interfacial Protein Concentration

The HpH samples showed greater Γ values than the HC ones, the degree of hydrolysis only affected the HpH samples where DH 6% was significantly lower than the rest (Table 2). This could be attributed to two causes. Firstly, the HpH emulsions showed a greater adsorbed protein concentration at the interface for DH 0 and 1.8% (Table 2). Secondly, and probably most importantly, the HC emulsions showed a Sv value that was twice as much as those from HpH emulsions.

Table 2. *Interfacial protein concentration, interfacial area and adsorbed protein concentration in the studied emulsions.*
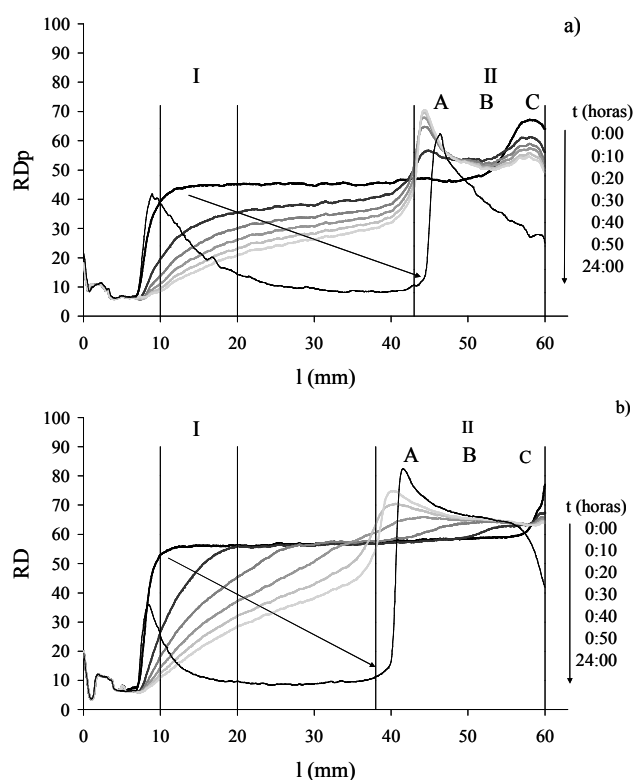
SPIH Stopping method and DH	Γ (mg /m^2)	Sv (m^2/ml emulsion)
HC-DH 0%	1.7 ± 0.1	0.29 ± 0.01
HC-DH 1.8%	1.5 ± 0.1	0.29 ± 0.01
HC-DH 2.5%	1.7 ± 0.1	0.29 ± 0.01
HC-DH 6.0%	1.3 ± 0.1	0.31 ± 0.01
HpH-DH 0%	4.2 ± 0.2	0.14 ± 0.01
HpH-DH 1.8%	4.2 ± 0.2	0.12 ± 0.01
HpH-DH 2.5%	4.2 ± 0.2	0.12 ± 0.01
HpH-DH 6.0%	3.3 ± 0.2	0.12 ± 0.01

3.1.3. Droplet Flocculation during Formation

The addition of SDS, according to[9], produces the dissociation of any type of aggregate that may have formed in the emulsions by non-covalent interactions. Therefore, the mean measured diameters ($D_{3.2}$ and $D_{4.3}$) in absence of SDS would indicate the droplets' tendency to flocculate through bridge formation. The variation of $D_{3.2}$ and $D_{4.3}$ values of the emulsions without SDS versus the different study variables, and the degree of flocculation of the studied emulsions is shown in Table1. The emulsions showed a modal distribution of the particle size (Figure 2) and great polydispersion, as observed in presence of SDS. $D_{4.3}$ is the most sensitive to droplet aggregation of all the measured mean diameters[17]. The emulsions prepared by HC ([protein] = 1 mg/mL) were the ones that showed the highest degree of flocculation of all the studied emulsion, particularly for DH 0, 1.8 y 2.5%, whereas those prepared with HpH under different assayed conditions did not show flocculation through bridge formation or flocculated only slightly (not more than 13%).

Figure 2. *Droplet size distribution (in volume) of the emulsions at pH 7.0 and [NaCl] = <0 M, with [protein] = 1 mg/mL of a) HpH-DH = 0% (▬) b) HC-DH = 0% (▪▪▪) y c) HC-DH = 6% (...). The measurements were performed in absence of SDS.*

3.2. Stability of the Emulsion

Figure 3. *Backscattering profile for oil-in-water emulsions a) SPIHC and b) SPIHpH. The arrows show the profile's change versus time. Time is represented on the scale on the right.*

Figures3A and B show the backscattering (BS) versus the height profiles of the sample tube corresponding to HC and HpH emulsions respectively, where [protein] = 1 mg/mL, pH 7.0 and [NaCl] = 0 M.

For both emulsions, the lower part of the sample tube, i.e. zone I (10 – 20 mm), shows a decrease in the RD values over time, reflecting the development of a creaming process, and in zone II the cream phase can be observed, which stayed after the oil droplets accumulated in the upper part of the tube.

3.2.1. Creaming Stability of the Emulsion

Figure 4 shows the variation curves of the meanBS values (BS_p) at the zone between 10-20 mm (BS_{p10-20}) versus time, corresponding to all the assayed conditions of the emulsions.

It can be noted than some curves show a hyperbolic shape (e.g. Figure 4a) whereas other show a sigmoid shape (e.g. Figure 4b)
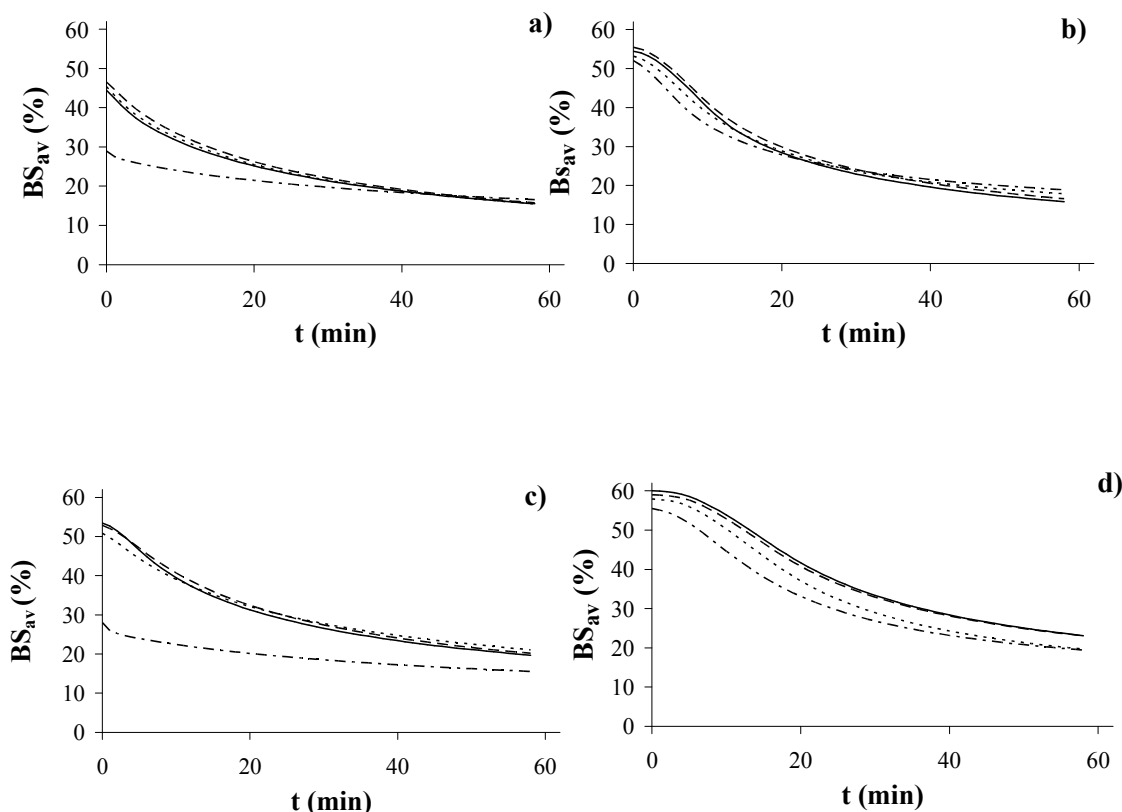
Figure 4. *Variation of the meanbackscattering values in zone 10-20 mm (BS_{p10-20}) versus time of the following emulsions a) HC emulsions [protein]: 1 mg/mL, b) HpH emulsions[protein]: 1 mg/mL, c) HC emulsions [protein]: 2 mg/mL and d) HpH emulsions[protein]: 2 mg/mL; DH = 0% (▬), b) DH = 1.8 (- -), c) DH = 2.5 (...) and d) DH = 6,0% (-··-).*

3.2.2. Creaming Stability Study

According to [14] emulsions with a hyperbolic BS profile would have such deviations from the Stokes' Law that, despite affecting the creaming rate of the droplets, these would keep the expected trend where larger droplets cream faster than smaller ones. Contrastingly, for emulsions with a sigmoid BS profile, the extent and incidence of the deviations from the Stokes' Law would be such that the movement of the droplets during the first interval of the creaming process would be impeded, however, over time, the delaying effects would decrease the extent and incidence, so that the creaming rate would assume a similar behavior to the hyperbolic profiles. The k_h value indicates the type of behavior regarding stability andk_s is an index of the delay of the creaming rate. The $BS_h$and BS_s values determinethe extent to which each term contributes to the overall destabilization process. In that respect, the BS_h/ BS_sratio is even more suitable [14].

Tables 3a and b show the k-values and %BS respectively, for the studied emulsions under all the assayed conditions.

HpH emulsions showed sigmoid-shaped BS vs t curves under every assayed condition, whereas HC emulsions showed hyperbolic curves under every assayed condition, except when the protein concentration was 2 mg/mL with DH 0, 1.8 and 2.5%, which had a sigmoid-shaped curve.

Emulsions with P =1 mg/mL showed no significant differences of Kh values, ks values and %BSs with different values of DH, whereas those with P = 2 mg/mL only showed a significant difference in kh, where the value corresponding to DH 6% was lower than the rest. This indicates that there was no influence of DH either on the rate or delay effect of the creaming process. When the protein concentration increased, the kh and ks values were significantly lower except for DH 6%, where no significant difference in the kh value was found, whereas the %BS values increased significantly. This indicates less tendency to cream and greater delay effect as concentration increases for HpH.

The HC emulsions [protein] = 1 mg/mL showed a hyperbolic kinetic behavior for all the studied DHs, with no significant differences in kh values. The increase in protein concentration cause a shift in the kinetic behavior (from hyperbolic to sigmoid) for DH 0, 1.8 and 2.5% and the kh values of those DHs did not show significant differences from each other and were considerably lower than the corresponding to DH 6%. In addition, the increase in concentration caused less of a tendency to creaming, since the ks values significantly decreased accordingly.

The way the hydrolysis was stopped significantly affected the creaming rate and the delay effect. The kh values corresponding to HpH emulsions were significantly lower than those of HC emulsions in every studied condition. The ks values of HpH were significantly lower than those of HC, while the %BSs were three time greater than those of HC.

The factors that could affect delay process of the creaming rate (as predicted by Stokes' Law) and manifest as a sigmoid

BS vs time profile are numerous:electrostatically charged droplets in the emulsion[18],[19], high effective density of the disperse phase [20], high apparent viscosities at low shear flow of the dispersing phase [21], [19], concentrated emulsions [22],[23], size and structure of the floccules within the emulsion [24],[25], [19], [26],[27] and polydisperse emulsions [28],[29],[30].

The surface electric charge, density and viscosity of the disperse phase were constant throughout the study, and therefore their delaying effect on the creaming rate must also have remained constant. The HC emulsions were those with the highest degree of flocculation and were also the only ones that showed a hyperbolic behavior. Then, even if the HC could have been forming floccules in an open-net structure, this would not have been able decrease the creaming process enough to generate a sigmoid behavior kinetics.

According to [27], creaming can be completely inhibited when a certain Φ_c value is surpassed, and this value depends on the polydispersion of the emulsion. Then, in the studied emulsions, the particle concentration could have been such that the effect of droplet concentration and polydispersion would not only have caused a decrease in the creaming rate, but it would have also delayed the movement of the larger droplets. The particle concentration and the droplet size interval decreased with the evolution of the creaming process, so that the degree of delay could become smaller while the creaming rate increased. The higher the particle concentration and degree of polydispersion, the greater the delay in the creaming rate was and hence the lower the k_s value was, which describes the sigmoid behavior of the RD(t) vs t curves. The principle behind the Turbiscan equipment is the multiple dispersion of light and the fact that the BS values increase as the particle size decreases and as the particle volume fraction increases[31]. In general, the emulsions which had a sigmoid-like kinetic behavior showed significantly higher $BS_{p(10-20)i}$ values than those emulsions with a hyperbolic behavior (Table 3). This agrees with the principle that the delay in the creaming rate depended on the droplet concentration and their size. Furthermore, the emulsions with a hyperbolic kinetic behavior had a tendency to cream from the start as aggregated floccules, something which probably went on during the creaming process and contributed to increase the creaming rate, thus counteracting other processes that would favor its delay.

Table 3. *K-values and %BS of the studied emulsions.*

a. k-values

SPIH Stopping method and DH	[prot.] = 1 mg/ml		[prot.] = 2 mg/ml	
	k_h	k_s	k_h	k_s
HC-DH 0%	$(1.3 \pm 0.08) \times 10^{-3}$		$(4.9 \pm 0.05) \times 10^{-4}$	$(1.7 \pm 0.05) \times 10^{-2}$
HC-DH 1.8%	$(1.1 \pm 0.05) \times 10^{-3}$		$(4.4 \pm 0.05) \times 10^{-4}$	$(1.6 \pm 0.05) \times 10^{-2}$
HC-DH 2.5%	$(1.2 \pm 0.04) \times 10^{-3}$		$(4.4 \pm 0.05) \times 10^{-4}$	$(3.1 \pm 0.05) \times 10^{-2}$
HC-DH 6.0%	$(1.6 \pm 0.05) \times 10^{-3}$		$(1.9 \pm 0.05) \times 10^{-3}$	
HpH-DH 0%	$(4.8 \pm 0.05) \times 10^{-4}$	$(5.4 \pm 0.05) \times 10^{-3}$	$(3.6 \pm 0.08) \times 10^{-5}$	$(2.3 \pm 0,08) \times 10^{-3}$
HpH-DH 1.8%	$(5.0 \pm 0.05) \times 10^{-4}$	$(5.4 \pm 0.05) \times 10^{-3}$	$(8.0 \pm 0.05) \times 10^{-5}$	$(3.1 \pm 0.05) \times 10^{-3}$
HpH-DH 2.5%	$(5.3 \pm 0.04) \times 10^{-4}$	$(7.3 \pm 0.04) \times 10^{-3}$	$(2.2 \pm 0.04) \times 10^{-5}$	$(2.3 \pm 0.04) \times 10^{-3}$
HpH-DH 6.0%	$(5.4 \pm 0.05) \times 10^{-4}$	$(7.6 \pm 0.05) \times 10^{-3}$	$(3.9 \pm 0.05) \times 10^{-4}$	$(4.8 \pm 0.05) \times 10^{-3}$

b. Ratios of BS_h and BS_S and BS_i.

SPIH Stopping method and DH	[prot.] = 1 mg/ml			[prot.] = 2 mg/ml		
	$\%BS_h$	$\%BS_S$	BS_i	$\%BS_h$	$\%BS_S$	BS_i
HC-DH 0%	1.00 *		44 ± 1	0.74 ± 0.04	0.26 ± 0.04	53 ± 1
HC-DH 1.8%	1.00 *		47 ± 1	0.78 ± 0.04	0.22 ± 0.04	53 ± 1
HC-DH 2.5%	1.00 *		45 ± 2	0.83 ± 0.04	0.17 ± 0.04	52 ± 1
HC-DH 6.0%	1.00 *		29 ± 2	1.00 *		36 ± 1
HpH-DH 0%	0.44 ± 0.01	0.56 ± 0.01	54 ± 1	0.32 ± 0.02	0.68 ± 0.02	60 ± 1
HpH-DH 1.8%	0.46 ± 0.01	0.54 ± 0.01	55 ± 2	0.39 ± 0.02	0.61 ± 0.02	60 ± 1
HpH-DH 2.5%	0.49 ± 0.01	0.51 ± 0.01	53 ± 4	0.26 ± 0.02	0.74 ± 0.02	58 ± 1
HpH-DH 6.0%	0.48 ± 0.01	0.52 ± 0.01	52 ± 5	0.33 ± 0.02	0.67 ± 0.02	55 ± 5

* In the case of HC (1 mg/mL), the curve is not sigmoid and is described by equation 5, consequently it has only one term and a single amplitude parameter, therefore its ratio is 1 and is reported in the $\%BS_h$ column.

3.2.3. Stability of the Emulsion to Flocculation and Coalescence

In Figures 3A and B, zone II shows that the cream phase remained after the oil droplets gathered-up on the top of the tube. In order to compare the cream phases of the different assayed emulsions, the BS vs. l(tube height) profiles shown in Figures 3A and B were divided into three zones: A, B and C, corresponding to the lower, middle and higher parts of the cream phase respectively. The profile of several emulsions, represented in the different studied conditions (Figures 5a -f), show a peak in zone A, which, according to[15] can be ascribed to the build-up of small droplets with low creaming rate. In addition, another prominent peak appears in zone C as a result of the presence of foam in the emulsions. In the middle zone (zone B) of the cream phase, the drop in the BS profile may be regarded as the decrease of the number of droplets, mainly generated by coalescence or coalescence together with a flocculation process [15].

When the emulsions are prepared with proteins as their

only emulsifying agent, in low concentrations and under static conditions, coalescence is a slow destabilization mechanism compared to creaming and flocculation [32]. Storing the emulsions under static conditions, at room temperature for 24 hours is considered to be enough to detect coalescence [15]. In the emulsions corresponding to Figures 5a, b and e, zone B (50 – 53 mm) shows a marked decrease

in the BS profile after 24 hours relative to the profile after the first hour of destabilization, whereas the emulsions corresponding to the conditions of Figures 5c, e and f, do not show this decrease. These results may indicate that the first group of emulsions could have experienced a considerable droplet size increase during the first 24 hours of storage.
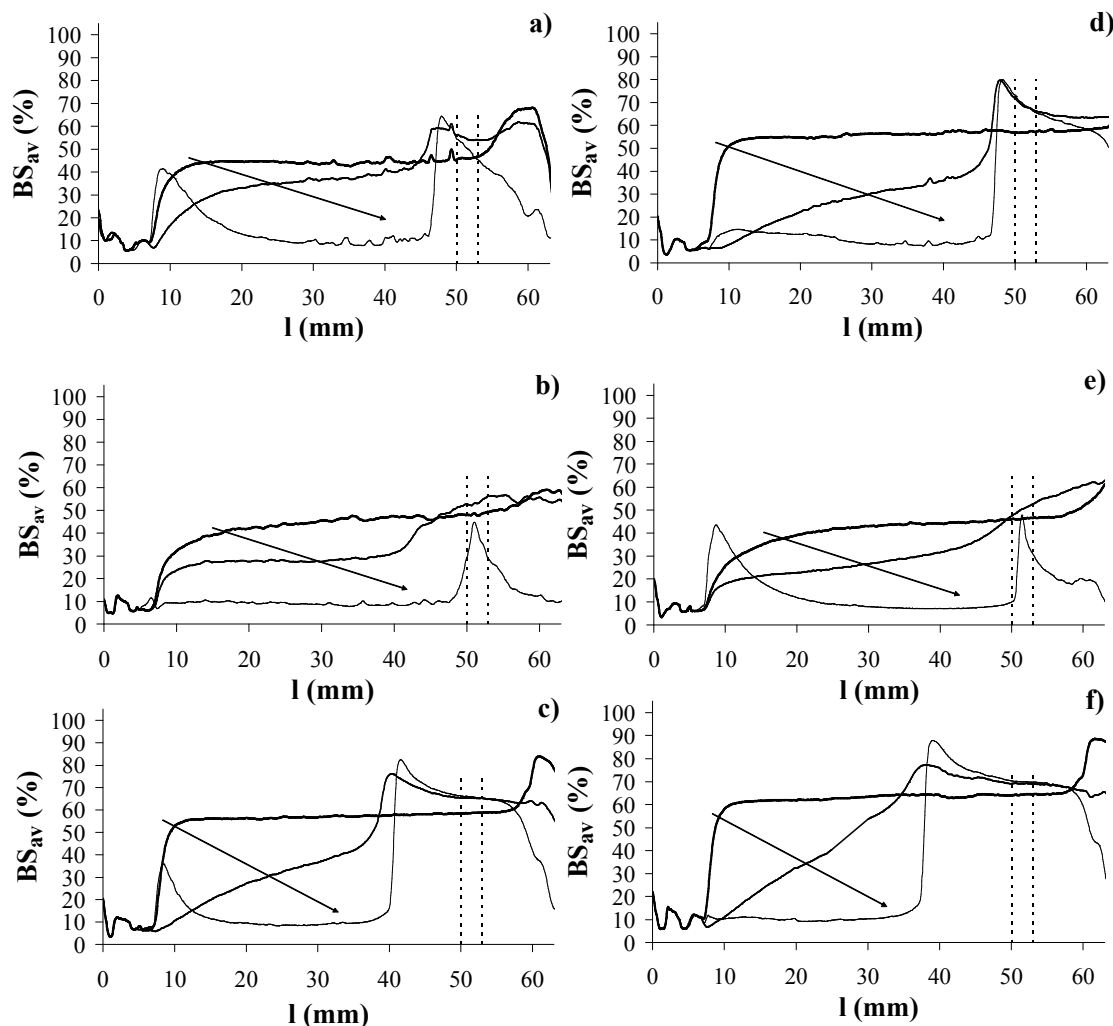

Figure 5. Backscattering profiles of emulsions prepared at 0 minutes, 60 minutes and 24 hours after the time of elaboration a) HC (DH = 0%, [protein] = 1 mg/mL), b) HC (DH = 6%, [protein] = 1 mg/mL), c) HpH (DH = 0%, [protein] = 1 mg/mL), d) HC (DH = 0%, [protein] = 2 mg/mL), e) HC (DH = 6%, [protein] = 2 mg/mL), f) HpH (DH = 0%, [protein] = 2 mg/mL. The arrow indicates the change of the profile over time. The indicated zone corresponds to the 50 – 53 mm interval.

3.2.4. Destabilization Kinetics of the Cream Phase

In order to analyze the destabilization kinetics of the cream phase, the BS_p in zone B (50-53 mm) taken from the BS profiles versus time (0 - 60 minutes) were recorded for all the assayed emulsions (Figures 6a-d). The time required for BS_p to reach the maximum value (BS_pmax.) is indicative of the balance between the build-up and decrease rates of the number of droplets. The lower values of BS_pmax., such as HC [protein] = 1 mg/mL, indicate that during that time the decrease in the number of droplets, due to coalescence and/or flocculation was greater than the build-up of droplets due to the creaming process. In the case of HpH emulsions, under the

most of the assayed conditions the maximum BS_p value was obtained at 60 minutes, which indicates a slow rate of formation of the stable cream phase. The two described situations are in line with the creaming destabilization behavior shown by both groups of emulsions.

The D(%) values (Equation 5) were obtained from the BS profiles. This parameter allowed the quantification of the overall destabilization of the cream phase since its formation until the end of its storage (24 hours). Since it is a BS_p decrease index, associated with a lower particle number, it can be associated with coalescence and/or flocculation.

The D% (BS_p50-53) values are shown in Table 4 for all the assayed conditions. The prepared emulsions, under the

different assayed conditions with HC, showed a considerable degree of destabilization, while those prepared by HpH were stable to flocculation and coalescence processes. The emulsions prepared byHC ([protein] = 1 mg/mL) had a prevalence of particle inflow into the cream zone, whether flocculated or not, causing an increase of BS_{p50-53}, peaking after 8 to 20 minutes. In the case of DH = 0, 1.8 y 2.5%, that value decreased as the degree of hydrolysis increased, whereas the degree of destabilization increased (Figures 5a, b y e). For DH 6% BS_{p50-53} it decreased from the beginning and it also displayed the highest D% value.

According to [33], the stability of oil-in-water emulsions is mainly affected by the viscoelastic properties of the surfactant film, and the steric repulsion between the adsorbed surfactant layers, which in turn delays flocculation in the initial stages that leads to coalescence. As stated by [27] the partially hydrolyzed do not prevent coalescence as efficiently since they tend to form less viscoelastic and thinner interfacial layers, which break more easily. This could account for the increase of the degree of destabilization as DH rises, to such extent that DH = 6% emulsions showed a decrease in BS_{p50-53} from the beginning. On the other hand, the probability of forming a breach in a given place of the membrane is higher as the area increases. Consequently, the greater the contact area between two droplets, the greater the coalescence rate. The coalescence rate therefore increases as the droplet size of an emulsion increases, or when the droplets are pushed against each other. This process takes place in very concentrated emulsions and cream layers [17], [19]. In the case of DH 6%, larger droplets formed from the start (Table 1) which subsequently favored coalescence.

The HpH proteins had more flexibility to get placed at the interface, since they were previously dissociated and denatured by the low pH, particularly the AB glycinin subunits. Additionally, having a greater interfacial protein concentration favored the steric repulsions and therefore made it less likely to flocculate. The greater interfacial protein concentration also provided more resistance to coalescence to the film. For DH 6%, despite the high loss of β-conglycinin, which accounts for most of the stability of soy protein-derived emulsions [2], the aforementioned interfacial behavior was not affected. Therefore, most of the stability could be attributed mainly to the existence of glycinin as AB subunits.

The emulsions can become stable to coalescence when the thickness of the continuous interstitial phase is enough to prevent contact between the droplet films [27]. For this reason, a greater interstitial aqueous phase in the cream phase could reduce coalescence to a minimum [15]. In the measurements tubes, the actual sample height of the emulsion was about 53 mm (7 mm belong to the lower tube cap), from which about 40 mm correspond to the initial aqueous phase (75%) and about 13 mm to the initial oil phase (25%). Thus the limit between both phases should theoretically be at 47 mm. In the BS_{p50-53}vs. time plots of the emulsions prepared by HpH (Figures 5c and f), the BS profile of the cream phase corresponding to 24 hours begins

at 40 mm, which indicates that the cream phase is highly hydrated, which could have contributed to the flocculation and coalescence stability. On the contrary, the profiles at 24 hours of the HC emulsions begin around 47 mm, which indicates a poor hydration of the cream phase corresponding to 24 hours. The HC emulsions with a protein concentration of 2 mg/mL, despite showing more destabilization as the degree of hydrolysis increased, had a significantly lower destabilization than those with a protein concentration pf 1 mg/mL. This lower tendency to flocculation/coalescence compared to [HAS-C] = 1 mg/mL can be ascribed to the fact that, quite likely, interfacial protein concentration was higher in this case and therefore the film was more resistant to coalescence due to prolonged contact.

The most notable difference in the formation of the studied emulsions was the lower $D_{3.2}$ values of the HC emulsions. The higher degree of flocculation of the droplets in HC emulsions could mainly be a consequence of hydrophobic interactions, since soy proteins cannot undergo marked conformational changes at the interface [34], leading to some hydrophobic areas oriented towards the aqueous phase, enabling the interaction with other film proteins in neighboring droplets. Nevertheless, the droplets corresponding to the HC DH 6% emulsions showed a lower degree of flocculation. During the formation of the emulsion, before the coalescence caused by collisions, flocculation is likely to occur and, depending on the resistance of the protein film, coalescence may or may not occur. Emulsions with DH6% containing SDS showed a significantly higher $D_{4.3}$ value (about three times higher) than the rest of the HC samples. This could be explained by their lower film resistance (due to a higher degree of hydrolysis) that rapidly turned flocculation into coalescence. One possible explanation for the differences is that the HC samples were adsorbed more rapidly and efficiently. However, the proteins of HpH samples were dissociated (lower molecular size), unfolded, and showed a higher surface H_o[35], therefore they should have been adsorbed more rapidly and efficiently (or at least similarly) than the HC ones, but definitely not more slowly. The droplets formed with HpH showed a higher interfacial protein concentration and, consequently, considering that, they should have been more resistant to collision-mediated coalescence that may have occurred during homogenization. All evidence would suggest that HpH should have shown a better behavior during the formation of the emulsions. However, based on observations to date, the lower $D_{3.2}$ of the HC emulsions could be a result of the formation of a membrane that is more resistant to coalescence, and this behavior could be linked to a higher interaction of proteins with the oil phase and with each other, conferring it with more cohesiveness.

On the other hand, in the cream phase the droplets of HC emulsions showed a remarkable degree of destabilization and a considerable degree of coalescence, while the HpH emulsions showed great stability and no coalescence was noted during the duration of the study. Although coalescence was a result of collisions and prolonged contact (in the cream

phase), it is conceivable that a membrane capable of resisting the coalescence phenomenon in one scenario should be able to resist it in the other. From this observation it follows that the differences between the $D_{3.2}$ of the HC and HpH emulsions should not be ascribed to the droplets' resistance to coalesce, given the collisions they experience during homogenization. One plausible explanation of the observed phenomenon is that whether a droplet is prone to break up during homogenization also depends on the emulsifying capacity to improve rheological and viscoelastic properties of the interfacial film, since it would make it less likely for the droplets to break up and give place to larger droplets than expected for a given equilibrium interfacial tension [36],[37],[38]. This is probably the case for droplets in HpH samples, which also agrees with their behavior in the cream

phase. In sum, there actually is agreement between the behavior of the droplets in the different emulsions with the formation, stability and properties that the proteins conferred to the interfacial films.

Table 4. *Degree of destabilization (D(%)) in the 50-53 mm zone.*

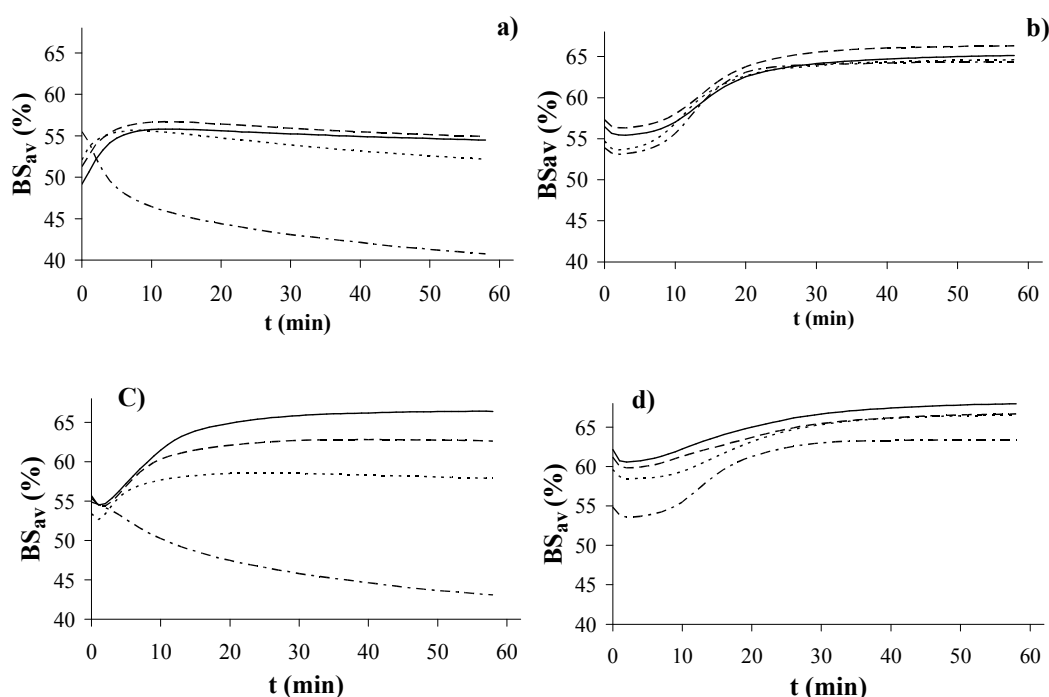| Stopping method of SPIH and DH	[prot.] = 1 mg/ml	[prot.] = 2 mg/ml
HC-DH 0%	13 ± 4	1 ± 1
HC-DH 1.8%	15 ± 3	9 ± 2
HC-DH 2.5%	21 ± 3	17 ± 3
HC-DH 6.0%	59 ± 9	47 ± 9
HpH-DH 0%	S	S
HpH-DH 1.8%	S	S
HpH-DH 2.5%	S	S
HpH-DH 6.0%	S	S

S: stable

Figure 6. *Variation of the meanbackscattering values in zone 50-53 mm (BS$_{p50-53}$) versus time for a) HC [protein]: 1mg/mL, b) HpH [protein]: 1mg/mL, c) HC [protein]: 2 mg/mL, d) HC [protein]: 2 mg/mL (DH = 0% (—), DH = 1.8 (- -), DH = 2.5 (...) and DH = 6.0% (-.-).*

4. Conclusions

The obtained results show that a very slight hydrolysis (DH 1.8 and 2.5%) does not have a beneficial effect on the emulsifying properties of those hydrolysates where the enzyme activity was stopped by freezing. Only the HC samples with DH 6% showed changes in their properties, which were actually detrimental. The detrimental effect of DH 6% on the emulsifying ability was reflected on the greater obtained droplet size ($D_{4.3}$) and on the stability at greater D% values. The emulsions that were prepared with HC DH 6% showed a greater tendency to coalesce due to the collisions that took place during formation, and through contact with the cream phase. In every case, the detrimental effect was ascribed to the loss of molecular size, which did not allow the formation of a film with suitable viscoelastic

and cohesive properties. In particular, these negative effects may be attributed to the hydrolysis of β-conglycinin compared to the hydrolysis of glycinin. In the case of emulsions then, β-conclycinin is the one that displays the best emulsifying properties.

The SPIHs where the enzyme activity was stopped by lowering the pH showed better emulsifying properties that those that were untreated. The emulsions derived from them were the most stable against creaming, flocculation and coalescence. In every case, the positive effect was ascribed to the dissociation and denaturalization of soy proteins by the pH treatment, which favored the formation of a film at the interface with good cohesive and viscoelastic properties. Moreover, the fact that these emulsions had higher $D_{3.2}$ and $D_{4.3}$ values was also correlated to the characteristics of the formed film. As previously mentioned a DH 6% had

detrimental effects on the properties, but this effect was not observed in the case of HpHs. Despite β-conglycinin dissociating due to the low pH treatment, in that case of DH 6% it underwent extensive hydrolysis which was responsible for the negative effect on the emulsifying properties. Consequently, those enhanced film properties which lead to superior functional properties can be attributed mainly to the dissociation of glycinin into its AB subunits and their denaturalization.

The method used to stop the papain hydrolysis reaction of the soy proteins in the isolates was a key aspect in the structural and emulsifying properties of the obtained products.

The hydrolysis did not have a beneficial effect on the emulsifying properties of the hydrolysates that were stopped by freezing. A 6% degree of hydrolysis negatively affected the emulsifying properties of the HCs.

Stopping the hydrolysis reaction by lowering to pH 2 made a significant improvement of the emulsifying properties of the isolates, for all the studied degrees of hydrolysis.

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