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Development of Novel Pea Protein-Based Nanoemulsions for Delivery of Nutraceuticals

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A novel pea protein-based emulsifier was used in the production of oil in water (O/W) nanoemulsions. The regions of kinetically stabilized O/W emulsions were individuated in a pseudoternary diagram containing different pea protein, sunflower oil, and water fractions. The stable O/W emulsion region was widened by the addition of NaCl to the aqueous phase or, to a more significant extent, by high pressure homogenization (HPH) processing. The HPH treatment caused the formation of very fine emulsions in the nanometric range (<200 nm), with extremely high stability over time. HPH treatment affected the protein properties, resulting in a reduced water surface tension. Changes in protein structure, which were characterized by light scattering and SDS-PAGE, suggest the occurrence of the disruption of disulfide bonds, thus changing the exposition and availability of hydrophobic groups.

KEYWORDS: Pea proteins; nanoemulsions; high pressure homogenization; surface tension; SDS-PAGE

INTRODUCTION

In recent years, the encapsulation of bioactive compounds into delivery systems, which can be easily incorporated into foods, has attracted significant attention from both scientific and industrial communities. The capsules can protect the bioactives from reacting with other food ingredients, thus efficiently preventing their degradation as well as any other alterations of the food quality and nutritional attributes (*1*). In addition, when the bioactive compound is lipophilic, encapsulation may considerably increase its solubility in water, which is of significant importance in both functional foods and drug formulation (*2*).

Among encapsulation systems, oil in water emulsions appear particularly suitable for food applications because they can be constituted by natural food grade compounds such as natural lipids and emulsifiers. Furthermore, when lipophilic active compounds are encapsulated in fats, their bioavailability after ingestion is enhanced (*3*). This is due to increased dissolution and solubilization by biliar and pancreatic secrections, increased intestinal permeability, reduced metabolism, and excretion activity (*4*) as well as reduced transport resistances through the aqueous boundary layer on the intestine walls (*5*). Finally, if the encapsulating emulsion is of nanometric dimension, the absorption process is further accelerated because it is direct and does not require intraluminal digestion (*6*).

Nanoemulsions are very fine emulsions made of lipid droplets of nanometric size (50−200 nm), dispersed in an aqueous phase, prepared by means of high energy comminution with the use of an adequate emulsifier at the water/oil interface (*7*). Nanoemulsions are kinetically stabilized by the very fine dimension of the droplets (*1*). The most efficient and high-throughput comminution system for the production of nanoemulsions is high pressure homogenization (HPH). Because of the high level of pressure applied to the process fluid (up to 300 MPa in commercial systems), elevated fluid-mechanical stresses are exerted within the homogenization valve on the suspended particles, contributing to reducing the emulsion droplet size below micrometric size (*7*, *8*) as well as to obtaining a significant reduction of the microbial load present in liquid foods (*9*).

To form a nanoemulsion, the choice of the emulsifier represents a key issue because both a high interface adsorption rate and good interfacial activity are required to reduce the extent of coalescence of newly formed surfaces as well as stabilize the nanosized droplets (*1*). While for food grade artificial surfactants, these properties can be tailored by molecular engineering, it is more difficult to find natural emulsifiers which are suitable for the production of nanoemulsions.

Although grain legumes have been consumed in many countries for thousands of years, until only recently was their regular intake acknowledged to have beneficial health effects (*10*). Furthermore, grain legumes are an important source of proteins, which are not only a source of energetic compounds such as amino acids but also functional ingredients with direct bioactive roles (*10*). In particular, vegetable proteins derived from grain legumes were used as animal protein substitutes which could lead to new types of novel foods with improved nutritional value, texture, and shelf life (*11*).

The functional properties of these vegetable proteins provide suitable emulsifiers for the preparation of food emulsions and also offer the advantage of using natural products together with the health-beneficial properties of the proteins themselves. For instance, the production of oil in water emulsions using vegetable

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Table 1. Essential Amino Acids Content of NUTRALYS Pea Protein (*28*)

component	mass fraction (g/100 g of protein)
cystine + methionine	2.1
histidine	2.5
isoleucine	4.5
leucine	8.4
lysine	7.2
phenylalanine + tyrosine	9.3
threonine	3.9
tryptophan	1.0
valine	5.0

emulsifiers to fully replace egg yolk offers several dietetic advantages (*12*, *13*). Although egg yolk is the most commonly used emulsifier in foods like mayonnaise or salad dressings due to its favorable organoleptic and functional properties (*14*), alternative, vegetable emulsifiers might contribute to decreasing cholesterol and fat contents as well as increasing microbiological stability (*15*). Several vegetable proteins with observed functional properties are derived from soybean (*16*), fava bean (*17*), lupin (*13*, *18*), or pea (*11*, *19*−*21*). More specifically, pea protein isolate is a natural vegetable extract with several nutritional qualities which include the absence of lipids, as well as appropriate physical and functional properties such as good solubility in water (*17*), high oil in water emulsifying power (*17*, *22*, *23*), stability at high temperatures, and good foaming capacity (*24*).

In this paper, the functional properties of a novel pea protein product are studied with the aim of producing stable nanoemulsion systems for the delivery of bioactive compounds. More specifically, the ability of the protein products to act as emulsifying agents and stabilize emulsions including nanoemulsions has been studied, with a pseudoternary phase diagram having been constructed. The effects of the high pressure homogenization (HPH) processing parameters (i.e., pressure and processing passes) on the emulsion droplet sizes as well as the eventual modifications of protein structure and functionality have also been investigated.

MATERIALS AND METHODS

Materials. Pea protein based emulsions were prepared in different compositions by changing the emulsifier/oil/water ratios in order to evaluate the kinetic stability of different compositions. Sunflower oil from Sagra (Italy) was used as lipid phase, while a natural pea protein, Naturalys F85M, a generous gift from Roquette, Italy, was used as emulsifier. In general, pea seed proteins are composed of two families of proteins, globulins (80−90% w/w), and albumins (10−20% w/w) (*25*). Globulins are made of two major proteins, legumin and vicilin, with an average ratio of approximately 1:1. In addition, a third globulin protein, convicilin, is also present in small quantities (*26*). Naturalys F85 M was extracted from dry pea (*Pisum sativum*), with an 85% protein content as described in **Table 1**.

Emulsion Preparation. Raw or primary emulsions were obtained by homogenizing water−pea protein−oil mixtures through high speed homogenization (HSH) using Ultra Turrax T25 (IKA Werke GmbH & Co., DE) equipped with a S25 N18 G rotor operated at 24000 rpm for 5 min at 4 °C. The energy for emulsification was delivered to the systems as inertial forces and shear stress by the rotor as a function of the rate of revolution. The smallest size of the disperse phase attainable by HSH was in any case larger than 2 μm (*8*, *27*). Primary emulsions were then reduced into very fine secondary emulsions (nanoemulsions) by high pressure homogenization (HPH) in a Nano DeBEE Electric Benchtop Laboratory Machine (Bee Int., USA). The operating conditions varied between 100 and 300 MPa pressure levels, and the number of homogenization passes varied from 1 to 20. The inlet temperature was set at 4 °C and the outlet temperature, which was increased by the pressure energy release, was rapidly reduced in a heat exchanger to 4 °C.

Screening of Emulsion Formulations. Nine different samples were initially prepared by mixing a highly concentrated solution of pea protein in water with oil of different proportions (from 1:9 to 9:1). Aqueous solutions of pea proteins were prepared at 16.7% pea protein concentration, slightly higher than the solubility of the protein powder (*28*). After homogenization by HSH, part of the sample was collected in a tube for stability investigation, while the other part was serially diluted in water and treated by HSH to generate the other points of the phase diagram, which were all sampled and stored at room temperature. After 24 h from preparation the state of the emulsified systems was visually evaluated and classified in physically unstable or stable systems.

Samples which resulted fluid enough to be sucked and pumped by the intensifier of the Nano DeBEE system, were further treated by HPH.

Particle Size Measurements. Two different particle size measurement systems were used for HSH-treated and HPH-treated emulsions due to the significantly different characteristic mean droplet size.

A photon correlation spectrometer (HPPS, Malvern Instruments, Malvern, UK) was used for the particle size measurement of the HPH-treated emulsions, whose characteristic size was always comprised in the instrument sensitivity range (1−6000 nm). The droplet size distribution was characterized in terms of the hydrodynamic diameter (d_H) or *z*-diameter, which was determined by cumulant analysis of the intensity−intensity autocorrelation function G(q,t) (*29*).

The particle size distributions (PSD) of the HSH-treated emulsions and of the pea protein suspensions were instead measured by laser diffraction (Mastersizer 2000, Malvern Instruments, Malvern, UK). In this case, the PSD was characterized in terms of the Sauter diameter $d_{3,2}$ and of $d_{4,3}$, whose mathematical expressions are reported in eqs 1 and 2, where x_i is the diameter of the single droplet and the sum is extended to the whole population.

$$d_{3,2} = \frac{\sum x_i^3}{\sum x_i^2} \quad (1)$$

$$d_{4,3} = \frac{\sum x_i^4}{\sum x_i^3} \quad (2)$$

Surface Tension Measurements. Surface tension was measured at the air−water solution interface, with different protein loadings in the solution, by means of the pendant drop method. A CAM200 apparatus (KSV Instruments, Finland), consisting of an experimental cell, an illuminating, and viewing system to visualize the drop as well as a data acquisition system, was used to determine the interfacial tension from the pendant drop profile. Images of the drop are captured and digitalized, and the drop contour is extracted for the determination of the radius of curvature at the apex necessary for the calculation of interfacial tension. Surface tension is automatically determined via a software by fitting the shape of the drop (in a captured video image) to the Young−Laplace equation which relates interfacial tension to drop shape.

Equation 3 reports the Young−Laplace equation, where ϕ is the angle made by the tangent at the point (*X*, *Z*), *s* is the linear distance along the drop profile, and $d\varphi/ds$ corresponds to the radius of curvature at the point (*X*, *Z*) and β is the shape parameter, given by eq 4, where *g* is the gravitational constant, σ is the effective density of the liquid drop, γ is the surface or interfacial tension, and *b* is the radius of curvature at the origin.

$$\frac{d\phi}{ds} = 2 + \beta Z - \frac{\sin\phi}{X} \quad (3)$$

$$\beta = -\frac{g\sigma b^2}{\gamma} \quad (4)$$

The reported results are the average of seven measurements.

SDS-PAGE Electrophoresis of Pea Proteins. The protein fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), following the procedure described by Laemmli (*30*).

30% Acrylamide:Bis solution (29:1), *N*,*N*,*N*,*N*-tetramethylethylenediamine (TEMED), prestained SDS-PAGE standards broad range, and Coomassie Brilliant Blue R250 were obtained from Bio-Rad Laboratories. Glycerol, trizma base, glycine, SDS, 2-β-mercaptoethanol, bromophenol

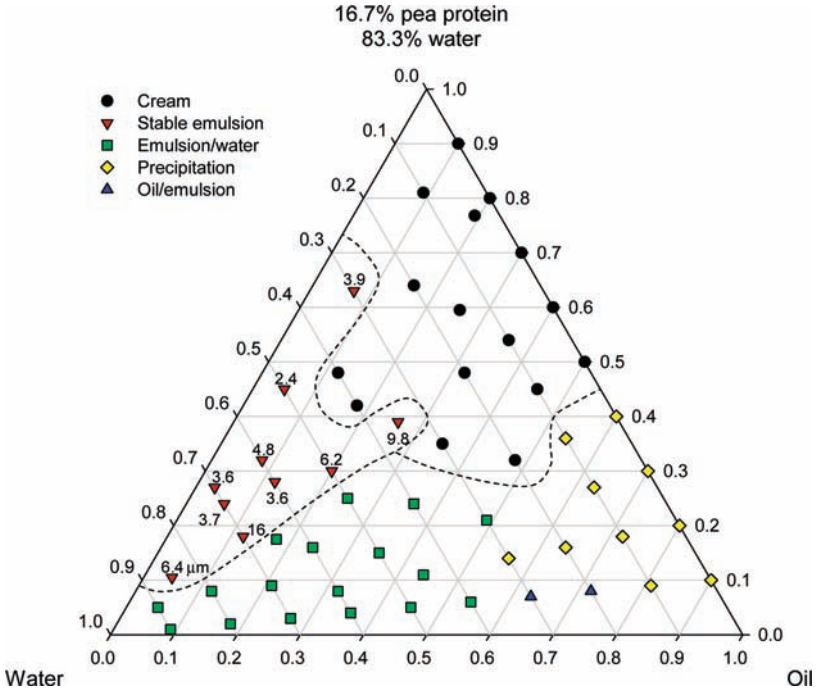

Figure 1. Pseudoternary phase diagram and stability of pea protein (F85M)−sunflower oil−water system homogenized by HSH. For stable emulsion formulations, the $d_{3,2}$ diameters of the droplets are reported in μm.

blue, methanol, glacial acetic acid, *n*-butanol, and ammonium persulfate were obtained from Sigma Aldrich.

The control and HPH-treated protein solutions (4% pea protein in water) were dispersed in a reducing buffer (containing 0.3 M Tris/HCl pH 6.8, 10% glycerol, 4% SDS, 10% 2-β-mercaptoetanol, 0.2% bromophenol blue) and denatured in a boiling water bath for 5 min.

The samples were analyzed under a nonreducing buffer (2-β-mercaptoethanol replaced by deionized water). A comparison of gels under reducing and nonreducing conditions made it possible to estimate the amount of protein involved in disulfide-bonded complexes.

The samples were then subjected to SDS-PAGE electrophoresis (*30*,*31*), using 4% polyacrilamide upper gel and 10% polyacrilamide lower gel. The electrophoresis was carried out using a Mini-system apparatus (Eppendorf AG, Germany) at room temperature, with an initial constant current of 40 mA. The electrophoresis current was increased after 1 h to 50 mA until the bromophenol blue reached the bottom. After electrophoresis, the gel was transferred to the staining solution, which contained 0.1% Coomassie Brilliant Blue R250 dissolved in methanol (50%)/glacial acetic acid (10%), and stored in the staining solution for 2 h. Finally, the gel was kept in the destaining solution overnight.

RESULTS

Stability of the Primary Emulsions. The emulsion-based nanometric delivery systems were prepared by high speed homogenization (HSH) for the formation of the primary emulsions, followed by high pressure homogenization (HPH) for the formation of the secondary nanoscale emulsions. The stability of the primary emulsions was evaluated for different formulations in terms of emulsifier and oil contents and reported in a pseudoternary phase diagram, as shown in **Figure 1**. Pseudoternary phase diagrams are frequently used, especially in the formulation of self-emulsifying drug delivery systems (*32*), which are thermodynamically stable emulsions. In this case, a pseudoternary diagram is used as a compositional map for the identification of the optimal conditions to obtain kinetically stable emulsions, through the reduction of the droplet size and minimization of the amount of emulsifier to be used. For this reason, in **Figure 1**, it is possible to find additional information, such as the mean particle sizes measured for the stable emulsions. In the triangular phase diagram of **Figure 1**, the first vertex corresponds to water, the second to sunflower oil, and the third to the protein−water solution in 1:5 ratios (16.7% pea protein and 83.3% water). Nine different samples were initially prepared by mixing only the emulsifier and oil at different ratios (from 1:9 to 9:1), which on the diagram are located on the right side of the triangle, and emulsified by HSH. The other points reported in the diagram are generated by the serial dilution of the original nine mixtures. After 24 h from preparation, the state of the emulsified system was visually evaluated and classified. The unstable systems include the "precipitation" case, characterized by the physical separation of the pea protein, which precipitated to the bottom of the tube, the "emulsion/water" case, where two layers were observed with excess water at the bottom and the emulsion on top as well as the "oil/emulsion" case, where excess oil floated on top of the emulsion. It has been observed that the unstable zone of the diagram is confined to its lower part, while the stable conditions all exist above the imaginary water dilution line that starts from the middle of the right side of the phase diagram and reaches the water vertex. Along this line, the pea protein to oil ratio is constant and corresponds to 1:6, which can be considered the minimum value to obtain a physically stable emulsion by HSH processing.

The stable systems that were obtained were divided into two large categories: the "cream" case, which included the emulsion systems with a macroscopically non-Newtonian behavior and the "stable emulsion" case, which included all the formulations diluted to the points that exhibited a Newtonian flow behavior. The "stable emulsion" systems were homogeneous and could be used in the preparation of delivery systems. They were considered to be the primary emulsions for further processing by HPH and comminution to nanometric size for the production of the secondary emulsions. Thus, on all the stable formulations experimentally found, the Sauter diameters $d_{3,2}$ were measured by laser diffraction and reported in **Figure 1**. In agreement with the data reported in current literature, a high speed homogenization system can generate emulsions with mean particle sizes above

Table 2. Composition and Sauter Diameter of NaCl-Stabilized Emulsions

oil [%]	pea protein [%]	water [%]	$d_{3,2}$ [μm] NaCl 50 mM	$d_{3,2}$ [μm] NaCl 150 mM
24.0	2.7	73.3	2.9	3.1
25.0	4.2	70.8	2.5	2.7
17.5	2.9	79.6	2.2	3.9

2 μm (*8*, *27*), with a larger size being obtained closer to the instability region.

The addition of NaCl to the aqueous phase was also investigated as a means to increase the stability region in the pseudoternary diagram by affecting the surface tensions of the oil and water phase. NaCl, which is considered to be a natural stabilizer, was added to the water phase at the concentration of 50 and 150 mM, within the limits defined for beverages (*1*), in different formulations around the stability region reported in **Figure 1**. The results show that the stability region is enlarged only above the pea protein to oil ratio of about 1:10. Below this ratio, the NaCl addition, at either 50 or 150 mM, does not contribute to the stability of the systems, although it is effective as an emulsifier to the oil ratio in the region between 5:5 and 4:6. **Table 2** shows the composition and Sauter diameters of the three different systems in this region.

No significant differences were observed among the samples with different NaCl concentrations, as shown in **Table 2**. The slight increase in mean particle size upon increasing NaCl concentration may be explained due to the enhanced coalescence when the salt concentration exceeds certain limits. This observation is coherent with previously reported results, which have shown that the addition of salt could significantly affect the viscosity and interdroplet interactions of protein-stabilized emulsions, but it did not influence their droplet size distributions (*33*). More specifically, the addition of salt may induce two opposite effects on emulsion stability. Protein-based emulsions can be partly destabilized by the reduction of electrostatic repulsion among the droplets, with the modification of hydrophobic interactions between nonpolar amino acids residues due to the alteration of the structural organization of water molecules at the interface (*34*). In contrast, NaCl can also promote an increase of effective adsorbed protein concentration to stabilize the film (*35*).

Whether the destabilizing or stabilizing effect predominates depends on the concentration of salt and/or ionic strength (*36*). At low ionic strengths, the influence that salt exerts on the protein structure is governed by electrostatic interactions. At higher ionic strengths, the stabilization of the protein structure is attributed to the preferential hydration of the protein molecule as a result of a salt induced alteration of the water in the vicinity of the protein (*37*).

Stability of the Nanoemulsions. On the basis of the pseudoternary stability diagram reported in **Figure 1**, the operative conditions for the production of nanosized emulsions by HPH processing were investigated. Screening investigations on the process parameters were conducted on the formulation comprising 6% oil, 4% pea protein, and 90% water. The $d_{3,2}$ of the primary emulsion obtained by HSH for this formulation was 3.7 μm (**Figure 1**). This emulsion was processed by up to 20 passes of high pressure homogenization at three different pressure levels (100, 200, and 300 MPa), and the mean droplet diameters were measured, as shown in **Figure 2**. The production of nanoemulsions should be considered successful when mean droplet sizes below 200 nm are reached. Below 200 nm, the passive mechanisms of absorption are activated due to the subcellular size of the emulsion droplets. **Figure 2** highlights that, in order to reach the threshold of 200 nm, 3 or 20 passes are required at 300 or 200 MPa, respectively. However, at 100 MPa, the mean particle sizes were never lower than 230 nm even after 20 passes. On the basis of these results, the processing conditions were defined and maintained at 300 MPa with five homogenization passes.

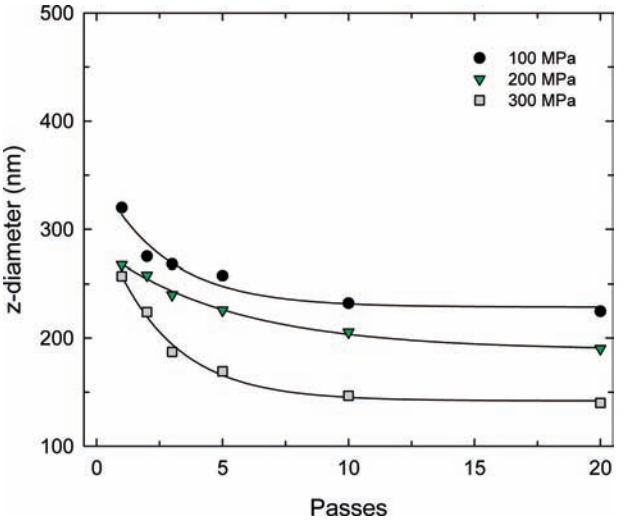

Figure 2. Evolution of *z*-diameter with pressure level and HPH number of passes for an emulsion with the composition of 6% oil, 4% pea protein, and 90% water.

The high pressure homogenization process results in the production of very fine emulsions with a narrow distribution of particle sizes, thus enhancing the kinetic stability of the systems. The consequence is that by HPH processing, it is possible to further widen the stability region in the pseudoternary phase diagram, making those kinetically stable formulations contain only a small amount of the emulsifiers.

In the case of the emulsifier tested in this work, the main advantage resides in the possibility to reduce its amount during the formulation of the protein-based emulsions, with a less significant impact on costs as well as the organoleptic properties of the products where the emulsions are incorporated.

Figure 3 shows how the HPH treatment, carried out on the noncreamy formulations, can alter the pseudoternary phase diagram and widen the stability region. Interestingly, stable formulations were also obtained for the pea protein to oil ratio lower than 1:6, in contrast with what was observed for HSH processing (**Figure 1**). Nevertheless, when considering the mean droplet sizes (*z*-diameters) reported in **Figure 3**, it is quite evident that the measured sizes did depend on the protein to oil ratios. In particular, when larger fractions of the emulsifier were used the droplet sizes were substantially independent from the composition. However, if less emulsifiers were used, droplets with larger sizes but minimum surface coverage of the oil droplets were obtained. These regions are exploded in **Figure 4**, where attainable particle sizes were reported as a function of oil and pea protein concentrations.

It can be postulated that during the secondary homogenization, the controlling step in the last region in determining particle size is represented by the fraction of emulsifier required to obtain surface coverage, so that when smaller droplets are formed, and hence with only partial surface coverage, they rapidly coalesce to form bigger particles until complete surface coverage is reached. Alternatively, in the region rich in emulsifiers, the final particle sizes are mainly determined by the efficiency with which the energy is transferred to the fluid. Because the processing conditions are

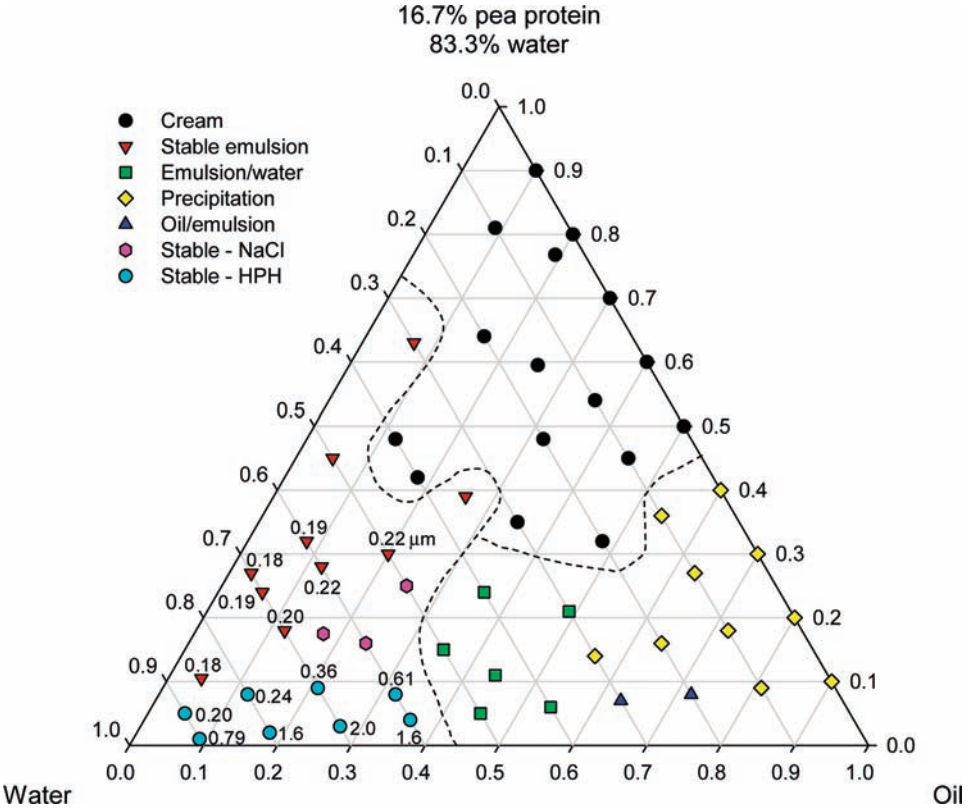

Figure 3. Pseudoternary phase diagram and stability of pea protein (F85M)−sunflower oil−water system homogenized by HPH (5 passes at 300 MPa). The *z*-diameters of the HPH treated emulsions are reported in μm.

always the same (5 HPH passes at 300 MPa), similar particle sizes are obtained in the submicrometer range (200 nm).

Figure 4 shows the mean droplet size of the emulsions, reported as *z*-diameter, obtained by HPH processing as a function of their composition (**Figure 4a**) as well as surface coverage and oil to protein ratio (**Figure 4b**). It is clear that the transition from micrometric emulsions to nanoemulsions occurs when the pea protein concentration in the emulsion is above a certain level, which depends on the oil concentration. This dependence is more explicitly showed in **Figure 4b**: it is not possible to obtain nanoemulsions (< 200 nm) when the surface coverage of the emulsion is below 5 mg/m^2, which corresponds to the minimum amount required to completely cover the oil surface at this dimension.

It is interesting to observe that, even when the surface coverage was increased from 5 to 40 mg/m^2, it is not possible to further reduce the droplet size. In this case, the minimum attainable diameter depends on the kinetics of the fragmentation rate and recoalescence rate, which in turn depend on the adsorption rate of the emulsifier at the newly formed droplet interfaces (*38*). Experiments not reported here showed that under the same processing conditions and with the same oil emulsifier ratios, the mean droplet size was reduced to 80 nm when Tween 80 was used as the emulsifier.

Protein Characterization upon HPH Treatment. The functional properties of the pea protein can be correlated to the effect of the hydrophilic emulsifier on water surface tension, which was characterized at different pea protein concentrations, homogenized with water by HSH (5 min at 24000 rpm), and reported in **Figure 5**. Because it is known that HPH treatment may affect the surface activity of proteins, the effect of different concentrations of pea protein on water surface tension was also measured after 5 HPH passes at 300 MPa. It must be highlighted that HPH treatment may have different effects on the properties of the protein when carried out in solution and in emulsion systems. The surface activity characterization was carried out in solution, while during emulsion preparation, the untreated proteins were subjected to HPH treatment in the presence of the emulsion droplets.

The results reported in **Figure 5** show that HPH caused a significant decrease in the surface properties of the pea proteins. In fact, water surface tension, as measured in air by pendant drop method, upon addition of 10% emulsifier is reduced from 74% to 65% when the water−protein system is HPH processed, in comparison to a reduction to about 50% after only HSH. Remarkably, the untreated pea proteins exhibit a strong effect on water surface tension already at low concentrations, apparently reaching the critical micelle concentration below 1.6% total protein concentration and above that, only moderately affecting the surface tension. From **Figure 5**, it is evident that from the initial value for pure water (74 mN/m), at 1.6% pea protein, the surface tension drops to 50 mN/m, and by further increasing the pea protein concentration, it is not significantly affected, decreasing to 47 and to 42 mN/m at 5.0% and 8.3% pea protein concentration, respectively.

In contrast, when the pea protein is HPH treated, a linear dependence of surface tension on the pea protein concentration can be observed, with a less steep initial decrease (**Figure 5**). At a 1.6% pea protein concentration, the surface tension is only reduced to 66 mN/m, and to 52 and 39 mN/m at a 5.0% and 8.3% pea protein concentration, respectively.

HPH processing undoubtedly affects the structure of proteins and therefore their surface activity. For example, **Figure 6** shows the particle size distributions of pea proteins before and after HPH treatment. The HSH-treated proteins exhibited a monomodal distribution of particle size, with a $d_{3,2}$ diameter of 4.4 μm and a $d_{4,3}$ diameter of 7.2 μm. In contrast, the HPH-treated

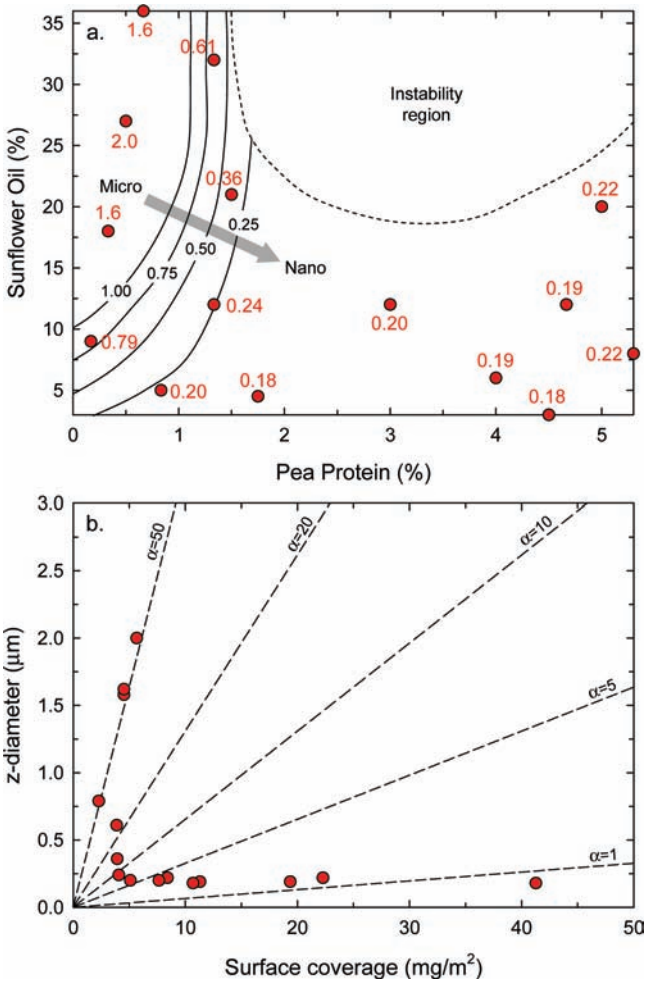

Figure 4. Mean droplet sizes (*z*-diameter, in μm) of the HPH-treated emulsions (**a**) as a function of oil and pea protein fractions, and (**b**) as a function of surface coverage of the emulsifier and oil to pea protein ratio α. HPH treatment consisted of 5 passes at 300 MPa.

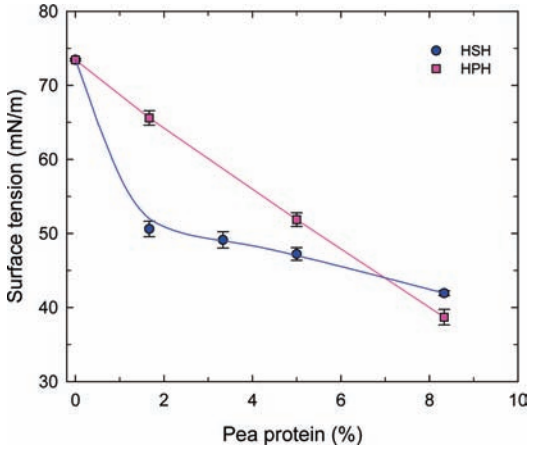

Figure 5. Surface tensions of water containing pea protein after HSH treatment (5 min at 24000 rpm) and HPH treatment (5 passes at 300 MPa), as measured by pendant drop method in air.

proteins exhibited a bimodal distribution of particle size, with a $d_{3,2}$ diameter of 2.7 μm and a $d_{4,3}$ diameter of 22.3 μm. In particular, shear phenomena associated with HPH processing induced macroscopic changes in protein agglomeration, with the formation of fine particles (10% of the particles were distributed

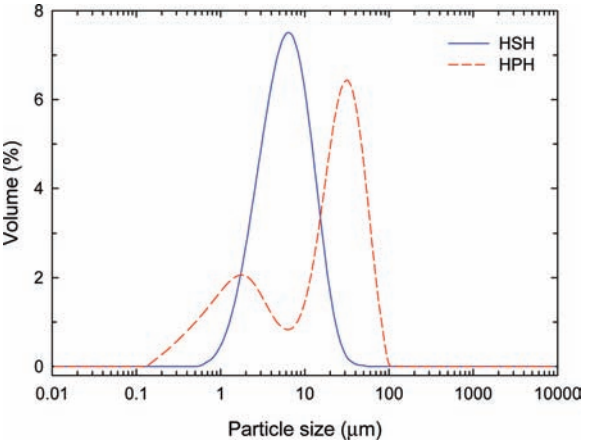

Figure 6. Particle size distributions of pea protein after HSH treatment (5 min at 24000 rpm) and HPH treatment (5 passes at 300 MPa).

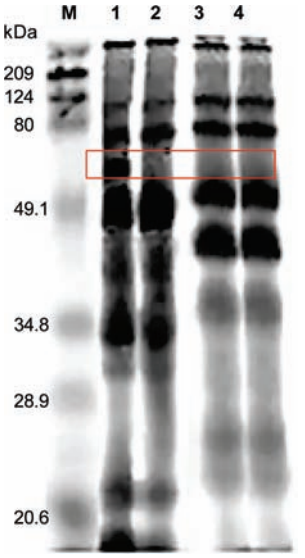

Figure 7. SDS-PAGE of protein samples after HSH treatment (lanes 1 and 3, 5 min at 24000 rpm) and HPH treatment (lanes 2 and 4, 5 passes at 300 MPa) under nonreducing (lanes 1 and 2) and reducing conditions (lanes 3 and 4). Reference (Lane M) was prestained SDS-PAGE standards of broad range.

below 0.6 μm in HPH treated samples while they were below 2.2 μm in HSH-treated samples) and aggregation in large globules (90% of the particles were distributed below 50 μm in HPH treated samples while they were below 14 μm in HSH-treated samples).

To clarify the nature of these agglomeration changes, SDS-PAGE tests were carried out on HSH and HPH treated proteins under nonreducing and reducing conditions.

The results reported in **Figure 7** suggest that HPH treatment induces a modification of the average molecular weight of the proteins. In fact, the SDS-PAGE results under nonreducing conditions (**Figure 7**, lanes 1 and 2) show that a significant change occurs in the HPH-treated sample, where a band between 49.1 and 80 kDa, highlighted in the box, disappeared. On the other hand, under reducing conditions (**Figure 7**, lanes 3 and 4), the pathways of HSH-treated and HPH-treated proteins became identical. Because the reducing agent used (2-β-mercaptoethanol) can disrupt disulfide bonds, it can be inferred that disulfide-bonded complexes, with a molecular weight between 49.1 and 80 kDa, are likely to be disrupted by the HPH treatment applied (5 passes at 300 MPa).

Previous characterizations of the effect of HPH treatment on emulsifiers were carried out for methylcellulose (*39*) as well as whey protein isolates (*40*, *41*). In both cases, and in contrast with what has been observed, HPH did not influence the interfacial tension of treated and untreated water−emulsifier solutions. Nevertheless, in the case of methylcellulose, it was reported that its average molecular weight was significantly decreased (*39*). Similarly, the effect of HPH treatment on whey protein isolates resulted in the irreversible disruption of the large protein aggregates into small entities, without affecting the protein solubility and conformation, as studied by SDS-PAGE (*40*). In addition, no denaturation was observed after the treatment for whey proteins. This observation was partly in contrast with isostatic high-pressure treatment of proteins, which have shown that pressure has marked effects on food protein functionality (*42*), primarily related to the rupture of noncovalent interactions within protein molecules as well as the formation of other intra- and intermolecular bonds (*43*). Nevertheless, the exposition time to high pressure in the HPH treatment is extremely short to promote such transformations, while shear stresses and high temperatures are more likely to be responsible for the protein transformation during HPH.

In contrast to the measurements obtained in this study, HPH treated whey proteins showed better stabilizing properties due to the increase of their surface hydrophobicity, even though the equilibrium interfacial tension resulted in being independent of the size of the proteins aggregates and therefore on the intensity of the HPH treatment (*40*).

On the basis of the results reported in **Figures 5**, **6**, and **7**, it can be postulated that the change in the functional properties of the pea proteins induced by HPH treatment can be attributed to the disruption of protein disulfide-bonded complexes, thus varying the exposition and availability of hydrophobic groups.

In summary, the study of pseudoternary phase diagrams as well as the stability of pea protein-based nanoemulsions showed the suitability of using a commercial pea protein as an emulsifier for the production of stable sunflower oil in water emulsions. Moreover, further processing by high pressure homogenization, despite the significant changes induced in proteins structure, with the disruption of some disulfide bonds and the consequent reduction of surface activities, produced extremely stable nanoemulsions, with a mean droplet size smaller than 200 nm, under composition ranges always characterized by protein to oil ratios smaller than 1.

Ongoing studies are focused on the application of the pea protein delivery systems for the encapsulation of vitamin E, by characterizing the physical and chemical stability of the active compound as well as its cell absorption in ex vivo studies.

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Supporting Information Available: Pseudoternary phase diagram. This material is available free of charge via the Internet at http://pubs.acs.org.

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