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High-Pressure Induced Physicochemical and Functional Modifications of Low-Density Lipoproteins from Hen Egg Yolk

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High-pressure treatment represents a potential method to stabilize microbiologically agricultural raw materials that are sensitive to heat treatments. Low-density lipoproteins (LDL), the main contributors to the exceptional emulsifying properties of yolk, are particularly sensitive to heat treatment. In this study, high-pressure treatments have been performed on LDL, and their impact on LDL physicochemical and emulsifying properties has been assessed. LDL dispersions at two pH levels (pH 3 and 8) were treated at different pressure levels: 200, 400, and 600 MPa at 20 °C. LDL dispersion characteristics (solubility, aggregation, and protein denaturation) and LDL emulsifying properties (o/w 30:70 emulsions: droplet size, flocculation, and protein adsorption) of nontreated and high-pressure treated dispersions were compared. Solubility is not altered by high-pressure treatment whatever the pH, whereas aggregation and protein denaturation are drastically enhanced, in particular at pH 8. The effects of these modifications on LDL emulsifying properties are mainly a diminution of the flocculation (depletion and bridging) at this same pH. Finally, it seems that high-pressure treatment combined with an alkaline pH decreases droplet flocculation of LDL dispersions.

KEYWORDS: LDL; high-pressure treatment; protein denaturation; protein aggregation; emulsifying properties

INTRODUCTION

Low-density lipoproteins (LDL) extracted from hen egg yolk are now confirmed as the principal contributor to emulsifying properties of yolk, by forming a film at the interface between oil and water (*1*). The characteristics of this film (organization, structure, composition, charge, viscoelasticity, and interactions) contribute to the physical stability of yolk emulsions. LDL (2/3 of yolk dry matter) are a globular supramolecular assembly of phospholipids and proteins (apoproteins) surrounding a neutral lipid core, and this assembly is essential to understand and control the production of food made with yolk, in particular, emulsions.

Concerning the adsorption mechanism of LDL at the oil−water interface, it has been supposed that LDL micelles break down when they come into contact with the interface. The lipid core coalesces with the oil phase, and proteins and phospholipids spread at the interface (*2*−*4*). The disruption of LDL particles is attributed to a weakening of the protein−protein interactions. We have recently confirmed the breakdown of LDL structures at the neighboring oil−water interface and the release and spreading of neutral lipids, phospholipids, and proteins (*5*). Consequently, we can hypothesize that LDL would act as a vector of surfactant constituents (hydrophobic proteins and phospholipids) that are water insoluble unless they adsorb at the oil−water interface. Then, the structure of LDL seems essential to ensure their interfacial properties, as any destructuring treatment affects their emulsifying properties.

Furthermore, Mizutani and Nakamura (*6*) demonstrated that extensive hydrolysis of LDL by proteases (trypsin and papain) produced a decrease of their ability to form and stabilize emulsions. It was suggested that only a small amount of the LDL phospholipids takes part in the adsorption at the oil−water interface and that the protein part of LDL played an essential role. We have confirmed these findings (*1*, *7*) by measuring the protein and phospholipid interfacial concentration in emulsions made with yolk, plasma, and granules (the two main fractions of yolk). We have observed that if the interfacial protein concentration was correlated with granulometry and stability of emulsions, the interfacial phospholipid concentration behaved very differently. In another way, Bringe et al. (*8*)

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noticed that emulsifying properties of yolk were not affected by the elimination of triglycerides and cholesterol from LDL. All these different results confirm the major role of the protein part.

Moreover, it has been demonstrated that yolk molecules, and predominantly LDL, are particularly sensitive to thermal treatments (*9*). Heat treatment (over 69 °C) of solutions mainly containing LDL (called plasma) causes a decrease in protein solubility and an increase in apparent viscosity. LDL contributes to both the decrease in protein solubility and the increase in apparent viscosity of plasma solutions because they have been shown to form strong cohesive gels when heated at 75 °C (*10*−*12*). Consequently, a rational demand exists to find microbial stabilization treatments of yolk solutions less denaturing than heat treatments. In this meaning, high-pressure processing is a promising technology to stabilize microbiological food products and could represent an alternative for eliminating microorganisms with a less detrimental physicochemical effect on yolk LDL. However, proteins or complex biopolymers subjected to high pressure may have their structure modified by a disruption of hydrophobic and electrostatic interactions (*13*). The occurrence of the denatured state of biomolecules depends on concentration, pH, pressure and temperature levels, and duration of the treatment. The structural modifications caused by high pressure on biomolecular complexes could bring about alterations of their functional properties (*14*) and particularly a reduction of emulsifying capacity (*15*). Concerning LDL, as we have stressed the importance of the LDL organization before adsorption, and of the protein part of LDL during adsorption, it seemed pertinent to examine the effect of high-pressure treatment on these two decisive characteristics.

Consequently, we have submitted dispersions of native LDL (10 mg of protein/mL) to three levels of pressures (200, 400, and 600 MPa) at two pH values (3−8) at 20 °C and measured physicochemical (solubility, aggregation, and thermal behavior) and functional (oil droplet size, flocculation, and protein adsorption) modifications.

MATERIALS AND METHODS

Preparation of LDL Fraction. Fresh hen eggs (Isabrown, France) were manually broken, and the yolks were rolled on a filter paper (Whatman, Maidstone, England) to remove the white and chalazes adhering to the vitellin membrane. The yolk membranes were then punctured, and the yolks were collected in an ice-cooled beaker. The yolk was diluted with an equal volume of a 0.17 M NaCl solution, and the yolk solution was equilibrated at 4 °C for 1 h. The pH was checked at 6.5. After centrifugation at 10 000*g* at 4 °C for 45 min, the supernatant (plasma) was collected, and the sediment (granules) was discarded. The plasma was centrifuged a second time under the same conditions for complete removal of granules. Purified plasma was then precipitated slowly with ammonium sulfate (40% saturation). The solution was stirred for 1 h before centrifugation at 10 000*g* at 4 °C for 30 min. The sediment (mainly γ-livetins) was discarded. The supernatant was dialyzed against deionized water for at least 6 h and then centrifuged at 10 000*g* at 4 °C for 30 min. The supernatant (LDL fraction) was stored at 4 °C for further analysis.

High-Pressure Processing. For high-pressure processing, dispersions of LDL of 10 mg of protein/mL at pH 3 (0.050 M glycine, 0.1 M NaCl) and pH 8 (0.050 M Tris-HCl, 0.1 M NaCl) were prepared.

High-pressure processing was carried out in a 3 L reactor unit (ACB Pressure Systems, Nantes, France) equipped with temperature and pressure control devices. Prior to pressure processing, 50 mL of LDL dispersions was vacuum packed in a polyethylene bag (La Bovida, France). The temperature during treatment was set at 10 ± 3 °C. Conditions of high-pressure processing were chosen in accordance to Chapleau and de Lamballerie-Anton (*16*). LDL dispersions (10 g of protein/L) were subjected to high-pressure treatment at 200, 400, and 600 MPa (±7 MPa) for 10 min. The level of pressure was reached at 6.5 MPa·s^{-1} and released at 20 MPa·s^{-1}. The temperature of the transmitting medium in the vessel was settled at 20 ± 2 °C during pressure processing.

LDL Solubility. Treated and nontreated (control = 0.1 MPa) LDL dispersions (10 mg of protein/mL) were centrifuged at 10 000*g* for 20 min at 20 °C. The supernatant protein content was determined by the Markwell et al. (*17*) method. Calibration was performed using standard bovine serum albumin (Sigma Chemical Co.). LDL protein solubility in percent was calculated as

$$\text{solubility (\%)} = \text{protein in the supernatant (mg/mL)} \times 100/\text{initial protein (mg/mL)}$$

Dynamic Light Scattering. The average diameters of protein particles of LDL dispersions (treated and nontreated) were measured by dynamic light scattering using a Log-Lin correlator (Malvern Instruments Ltd., Malvern, Worcester, UK). Measurements of the dynamic of scattered light were made at 90°, and average diffusion coefficients were determined by the method of cumulants. Particle diameters were calculated from diffusion coefficients using the Stoke−Einstein relation for spheres (*18*).

Differential Scanning Calorimetry. Differential scanning calorimetry was performed on a Micro DSC III (SETARAM, Caluire, France). Nontreated and pressured LDL dispersions (0.8 mL of a 50 mg of protein/mL dispersion) at pH 3 and 8 were heated in the calorimeter from 30 to 110 °C at 1 °C/min. A second scan was carried out on samples to check denaturation reversibility. Samples were hermetically sealed in iron pans. A sample buffer was used as a reference. Enthalpies of thermal denaturation (ΔH) were estimated as the area under the DSC curve. The enthalpy and thermal denaturation (Td) values of each peak was calculated by Micro DCS III software and expressed, respectively, as J/g of protein (dry matter basis) and °C.

Emulsion Preparation. Oil-in-water emulsions (40 mL) were prepared with sunflower oil and LDL dispersions (10 mg of proteins/mL) at pH 3 and 8 with an oil volume fraction (ϕ) of 0.3. The two phases were premixed for 30 s at 20 000 rpm with a polytron PT 3000 (Kinematica, Switzerland) equipped with a 12 mm diameter head. Homogenization of emulsions was carried out with a high-pressure valve Stansted FPG 7400 homogenizer (Stansted Fluid Power Ltd., Stansed, UK) at 120 bar with a recirculation of 3 min (*19*).

Average Droplet Diameter. Immediately after homogenization, 0.5 mL of emulsion was taken and diluted in 11.5 mL of 0.05 M pH 8 Tris-HCl buffer with 1% SDS. The droplet size distribution was estimated by laser light diffraction (λ = 658 nm) in a High Definition Particle Size Analyzer (Saturn DigiSizer 5200, Micrometrics Instrument Corporation, Atlanta, GA) and determined using the $d_{4,3}$ index. $d_{4,3}$ represents the mean diameter expressed in volume.

Flocculation Behavior. *Flocculation Index (FI).* The FI was calculated by the ratio between the $d_{4,3}$ droplet size in the buffer without SDS and the $d_{4,3}$ in the 1% SDS buffer (*19*):

$$\text{FI} = d_{4,3} \text{ of flocs}/d_{4,3} \text{ of droplets}$$

Depletion Flocculation. The depletion flocculation phenomenon can be analyzed by steady-state flow measurements through viscosity versus shear stress assays. A first step consisted of deformation of flocs present in the emulsion by application of a shear stress of 10 Pa during 2 min. Flocs become aligned with the shear field, which decreases their resistance to flow. Thereafter, the emulsion was left for 15 min to enhance droplet interactions. Finally, an increasing shear stress from 0.002 to 10 Pa in a 6 min period was applied to the emulsions for floc disruption (*20*). Measurements were performed at 20 °C in a Haake Rheostress RS75 equipped with a plate/cone sensor system (60 mm diameter, 2.09° angle).

Protein Adsorption. The oil droplets were washed from nonadsorbed proteins according to the method described by Patton and Huston (*21*). Two mL of fresh emulsion was diluted in 2 mL of sucrose solution (500 mg/mL in 0.1 M pH7 Tris-HCl buffer). Two mL of this mix was carefully deposited at the bottom of a centrifuge tube

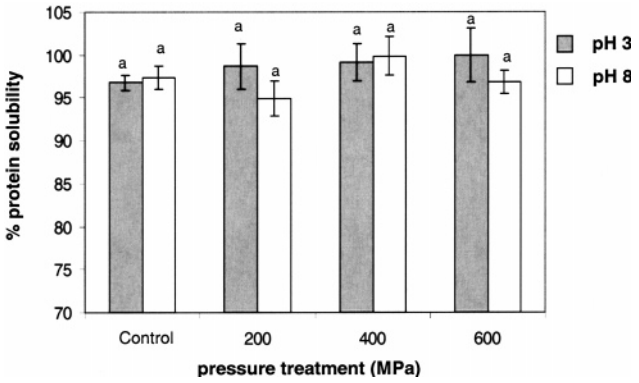

Figure 1. Effect of HP on protein solubility of pH 3 and 8 LDL dispersions (10 mg of protein/mL). Measurements were repeated 3 times. Means with same letters are not significantly different ($p < 0.05$).

containing 10 mL of the sample buffer solution. The tubes were centrifuged at 3000*g* during 2 h at 10 °C. After centrifugation, two phases were observed: the creamed oil droplets at the top of the tube and the aqueous phase of the emulsion at the bottom. The tubes were frozen at −20 °C and then cut so as to separate the phases. Adsorbed proteins at the creamed phase were desorbed by adding 20 mL of 1% SDS, and the dispersion was then centrifuged at 10 000*g* for 20 min at 10 °C.

The bottom aqueous phase, which contained the nonadsorbed protein, was carefully extracted with the aid of a micropipet. The adsorbed and nonadsorbed protein concentration of emulsions was determined by the method used by Markwell et al. (*17*). The percentage of adsorbed protein was calculated as

$$\%\ \text{adsorbed proteins} = \text{adsorbed protein concentration (mg/mL emulsion)}/\text{total protein concentration (mg/mL emulsion)} \times 100$$

Protein Composition of the Interfacial Film. The nature of adsorbed and nonadsorbed proteins at the interfacial film was analyzed by SDS−polyacrylamide gel electrophoresis. Continuous and stacking gels of 10 and 3.5% of acrylamide, respectively, were prepared. A buffer system containing 2 M Tris-base, pH 8.8 containing 0.15% SDS for the separating gel and a 0.027 M Tris-base, 0.38 M glycine pH 8.3 with the addition of 0.15% SDS for the running buffer were used. Coomassie Brilliant Blue was used as the colorant agent. Low MW markers (Biorad SDS-calibration kit) used included phosphorylase *b* (94.4 kD), bovine serum albumin (66.2 kD), ovalbumin (45.0 kD), carbonic anhydrase (31.0 kD), and soybean trypsin inhibitor (21.5 kD).

Statistical Analyses. Three measurements were conducted for the following characteristics: LDL solubility, dynamic light scattering, differential scanning calorimetry, average droplet diameter, flocculation index, rheology, and % adsorbed proteins. Statistical analysis was performed using a one-way analysis of variance according to the general linear model procedure with least-squares mean effects to determine significant differences between treatments. Multiple range tests were applied to determine which means were significantly ($P < 0.05$) different according to Fisher's least significant differences (LSD). Statistical analysis was carried out using Statgraphics plus version 2.1 software (Statistical Graphics Corp., Princeton, NJ).

RESULTS

Characterization of LDL Dispersions. *LDL Solubility.* Nontreated LDL presented a high solubility (>95%) whatever the pH (**Figure 1**). With a density close to that of water (0.98) (*22*), LDL does not sediment on centrifugation. This point is relevant as it means that LDL particles do not precipitate and hence are available, whatever the medium conditions, to participate at the formation of an interfacial film between oil and water.

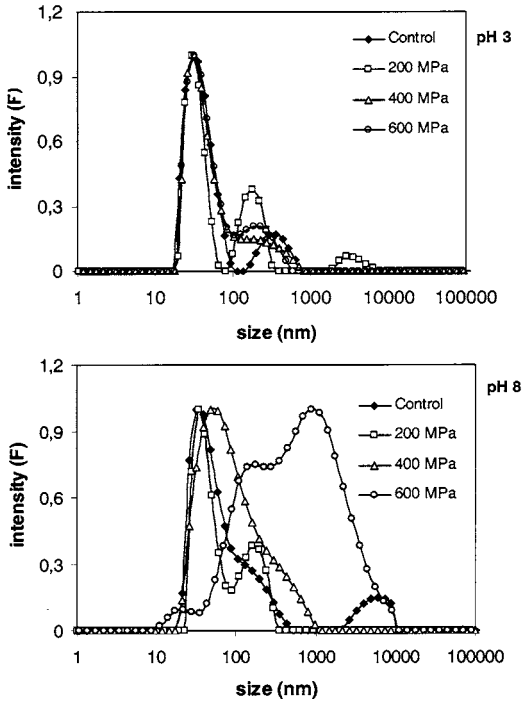

Figure 2. Dynamic light scattering of pH 3 and 8 LDL dispersions (10 mg of protein/mL). Measurements were repeated 3 times.

We can observe that, whatever the pH, high-pressure treatment has no significant effect on LDL solubility. We notice meanwhile a slight (but not significant) tendency to an increase of solubility at pH 3.

LDL Aggregation. Dynamic light scattering was performed in view of assessing the aggregation state of LDL due to high-pressure treatment, by the measure of the apparent hydrodynamic diameter. For the nontreated (control) LDL, at pH 3, two defined populations with different average diameters were observed (30 and 300 nm) (**Figure 2**). These two populations were also observed for nontreated LDL at pH 8. However, at this pH, we can observe another population of large aggregates: 6 μm. These results are in close agreement with the typical values found in the literature by dynamic light scattering and electron microscopy. Martin et al. (*22*) gave diameters ranging from 17 to 60 nm. Furthermore, concerning the structures of about a 300 nm diameter, it is likely that these structures are very low-density lipoproteins as observed by Martin et al. (*22*) or merged LDL as noticed for human plasma LDL (*23*).

Applied at pH 3, high-pressure treatment did not change drastically the diameter of the populations of LDL. However, at pH 8, the effect of high pressure on LDL aggregation was very important. We observe a progressive shift of the size of the first population from 30 to about 200 nm when the pressure increased up to 600 MPa. We observe also a shift of the second population from 300 nm for nontreated LDL and 200 Mpa treated LDL to 350 nm for 400 MPa and 900 nm for 600 MPa treated LDL. It seems that 400 MPa is the first level that really initiates a consequent aggregation of LDL, especially at pH 8.

Thermal Behavior. Thermograms of nontreated and high-pressure (HP) treated LDL at both pH values have permitted us to calculate the enthalpies of thermal denaturations (**Figure 3**). At pH 8, high-pressure treatment above 200 MPa induces a significant decrease of denaturation enthalpy without changes in denaturation temperature (results not shown) that was maintained at values around 70 °C. The enthalpy values

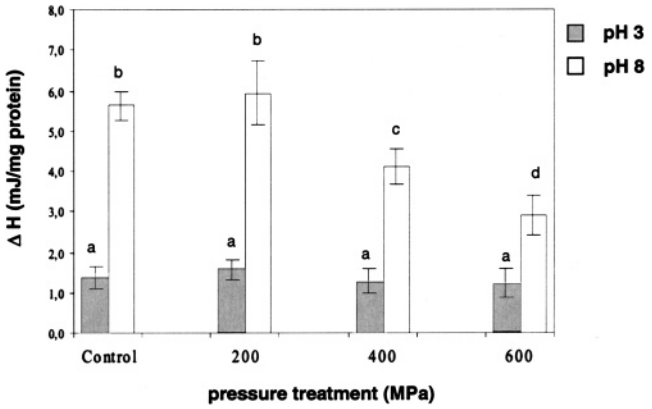

Figure 3. Effect of HP on denaturation enthalpy of pH 3 and 8 LDL dispersions (50 mg of protein/mL). Measurements were repeated 3 times. Means with same letters are not significantly different ($p < 0.05$).

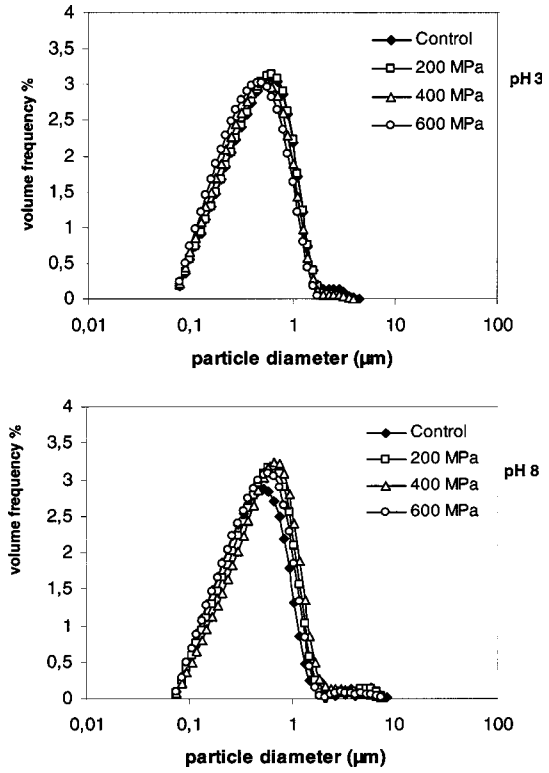

Figure 4. Volume frequency of emulsions ($\phi = 0.3$) prepared with pH 3 and 8 LDL dispersions (10 mg of protein/mL) in the presence of SDS. Measurements were repeated 3 times.

decreased by about 30 and 50%, respectively, for 400 and 600 MPa. This result suggests that LDL is partially denatured by the treatment. At pH 3, we can observe a great denaturation of control LDL due to the pH effect (≅70% as compared to pH 8). No effect of HP treatment in the denaturation enthalpy of LDL samples was observed nor in the denaturation temperatures.

Characterization of LDL Emulsions. *Droplet Size Distribution.* Droplet size distributions (calculated in volume) of oil−water emulsions made with nontreated and treated (200, 400, and 600 MPa) LDL samples are shown in **Figure 4**. We notice that whatever the pH and the pressure treatment of LDL, emulsions presented a monomodal distribution with a $d_{4,3}$ of about 0.44 (**Table 1**). All the emulsions were characterized by a distribution of oil droplets in the range of 0.1−5 μm. It seems that previous high-pressure treatment of LDL does not alter their capacity to stabilize freshly formed oil−water interfaces.

Table 1. Droplet Size ($d_{4,3}$), Flocculation Index (FI), and Percentage of Adsorbed Proteins of Emulsions Prepared with LDL Dispersions[a]

	$d_{4,3}$ (μm)	FI	% adsorbed proteins
		LDL pH 3	
0.1 MPa	0.46 ± 0.07 a	15.5 ± 1.8 a	66.9 ± 1.4 a
200 MPa	0.46 ± 0.06 a	14.4 ± 2.8 a	67.2 ± 1.7 a
400 MPa	0.42 ± 0.07 a	19.3 ± 2.3 a	67.1 ± 2.1 a
600 MPa	0.43 ± 0.06 a	18.7 ± 1.8 a	67.0 ± 1.6 a
		LDL pH 8	
0.1 MPa	0.42 ± 0.08 a	14.6 ± 1.4 a	67.3 ± 1.6 a
200 MPa	0.45 ± 0.05 a	13.3 ± 1.7 a	67.0 ± 2.2 a
400 MPa	0.48 ± 0.04 a	11.2 ± 2.2 a	67.4 ± 1.8 a
600 MPa	0.44 ± 0.04 a	3.0 ± 0.8 b	67.2 ± 1.7 a

[a] Measurements were repeated 3 times. Means with same letters are not significantly different ($p < 0.05$).

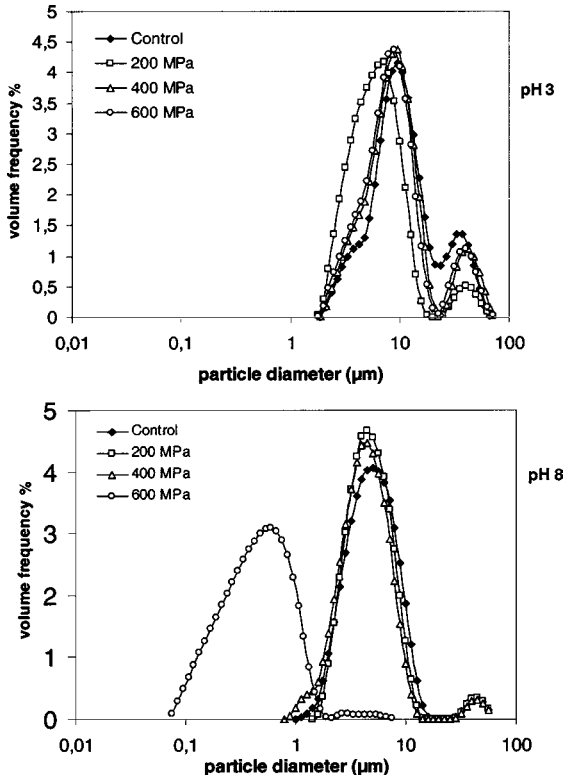

Figure 5. Volume frequency of emulsions ($\phi = 0.3$) prepared with pH 3 and 8 LDL dispersions (10 mg of protein/mL) in the absence of SDS. Measurements were repeated 3 times.

Flocculation Behavior. *Bridging Flocculation.* Without a deflocculation process (without SDS addition), emulsions made with nontreated LDL at pH 3 presented a droplet size distribution with a great percentage of particles of 8−9 μm and a minor fraction of particles of 35 μm (**Figure 5**). The appearance of these two higher populations without SDS addition suggests the presence of flocculated oil droplets in all the emulsions prepared with LDL. As depletion cannot occur due to the agitation motions in the granulometer cell, it is likely that bridging flocculation of droplets was observed in these emulsions. With a previous high-pressure treatment of LDL, the distribution was not significantly modified at acidic pH.

Emulsions prepared with LDL at pH 8 and nontreated and 200 and 400 MPa treated LDL presented similar droplet distribution without SDS, suggesting no changes in bridging flocculation at these HP levels. On the other hand, emulsions made with LDL treated at 600 MPa exhibited a monomodal

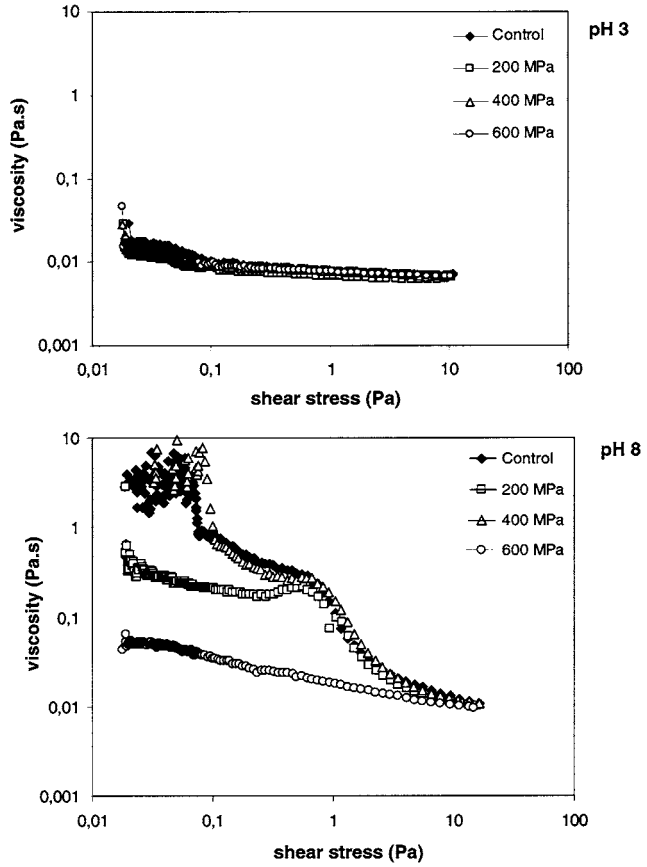

Figure 6. Viscosity vs shear stress of emulsions ($\phi = 0.3$) prepared with pH 3 and 8 LDL dispersions (10 mg of protein/mL). Measurements were repeated 3 times.

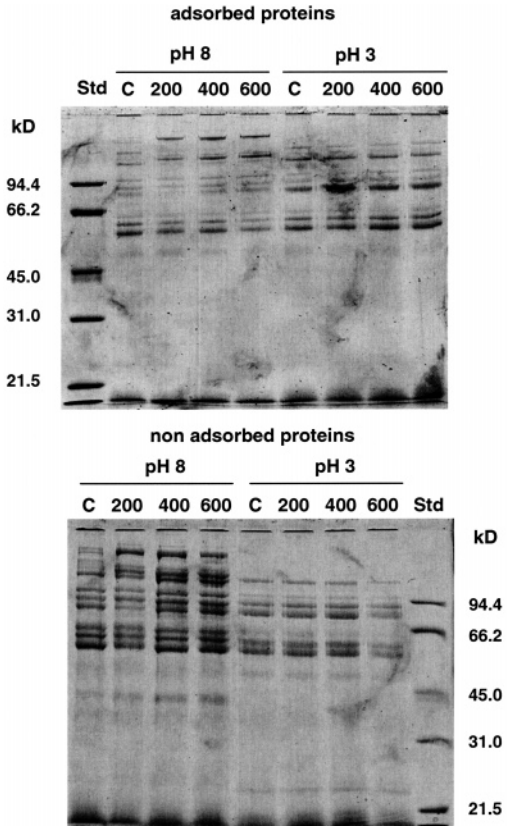

Figure 7. SDS–PAGE profile of adsorbed and nonadsorbed protein species at the oil–water interface LDL of pH 3 and 8. Std: standard; C: control sample; and HP: 200, 400, and 600 MPa.

distribution similar to that obtained with SDS ($d_{4.3} \cong 0.45$), signifying that in this case the emulsion was not flocculated.

On the basis of these results, we have calculated a flocculation index (FI) presented in **Table 1**. For LDL at pH 3, the FI values were not significantly modified by high-pressure treatment. On the other hand, for emulsions made with LDL at pH 8, FI values, stable between nontreated and 200 and 400 MPa, decreased drastically at 600 MPa of pressure treatment. A decrease of about 80% as compared to nontreated LDL was observed. This reflects the decrease of floc sizes noticed on the granulometric profiles.

Depletion Flocculation. Figure 6 shows the viscosity of emulsions as a function of shear stress application. The principle of the measure was first to eliminate any flocculation (bridging and depletion) by using an intense strain. Then, the reformation of droplet aggregates is highly influenced by depletion. Modification of emulsion flocs can then be characterized by viscosity variation measurements.

We can observe that, at pH 3, emulsions made with LDL presented no flocculation whatever the previous high-pressure treatment of LDL. These emulsions have a very low viscosity (0.01−0.05 Pa s) all over the range of shear. This behavior demonstrated the absence of depletion flocculation. Acidification changes LDL characteristics, leading to emulsions not able to develop depletion flocculation phenomenon.

At pH 8, emulsions made with nontreated LDL exhibited a high viscosity (10 Pa·s) at low shear stress and then a rapid drop of viscosity as the shear stress increased (from 0.1 to 0.2 Pa). A second drop was noticed from about 1 Pa. Then a complete disruption was observed from 10 Pa. The high apparent viscosity observed at low shear stress demonstrated the presence of a flocculated system. The initial viscosity is dependent on the size of droplet aggregates and, consequently, on the number of droplets concerned with these aggregates. Furthermore, the stress value corresponding to the drop of apparent viscosity gave an estimation of the applied strength to break the flocs.

High-pressure treatment of LDL at pH 8 did not drastically change this behavior until 400 MPa. But from 600 MPa, we can observe a quasi disappearance of the initial viscosity of emulsions, suggesting clearly a decrease of the depletion flocculation level due to the effect of pressure on LDL.

Protein Adsorption. We can observe in **Table 1** that the percentage of adsorbed proteins was not different whatever the pH used and that the high-pressure treatment did not alter the quantity of protein adsorbed at the oil−water interface. As there were no statistical differences between the droplet size of the different emulsions, we can assess that all the emulsions presented a similar concentration of adsorbed proteins (0.75 mg/m^2: not shown).

The nature of proteins (nonadsorbed and adsorbed) was analyzed by SDS−PAGE under reducing conditions (**Figure 7**). At pH 8, nontreated LDL (control: C) presented the typical profile of LDL proteins. We identified nine major bands of approximately 140, 100, 95, 85, 75, 65, 60, 55, and 15 kD. We can notice that acidification at pH 3 provoked the disappearance, previously reported (*24*), of the band of 140 kD. Application of HP treatment to LDL dispersion does not seem to favor the adsorption of any particular polypeptides.

DISCUSSION

HP Emphasizes Aggregation and Protein Denaturation at pH 8. The main LDL modifications by high-pressure treatment at pH 8 consist of a denaturation of proteins and a

subsequent aggregation. We have previously measured by transmission electron microscopy (*5*) that high-pressure treatment (from 400 MPa) provoked a disruption followed by an aggregation of LDL. The present study confirms these findings and determines the size of the aggregates. Furthermore, concerning the aggregation mechanism, our study suggests that high-pressure treatment provokes first a denaturation of LDL protein, leading to a disruption of the entity and then a reorganization by way of aggregation between proteins. Studies concerning the impact of heat treatment (freezing−thawing and heat) on LDL have revealed the disruption of the LDL structure during these treatments (*25*). This disruption is favored by dehydration in the case of freezing−thawing or by unfolding under heating. Lipid−protein interactions are weakened under freezing or heating, and interactions between proteins are increased. These interactions are both principally of a nonpolar nature because an LDL gel is solubilized by SDS, which interacts with the hydrophobic residues of proteins (*26*). The aggregation product of LDL certainly contains lipids included in the structure (*12*). Proteins of LDL present a large proportion of hydrophobic amino acids and, consequently, have a high ability of interacting by this way. Heat treatment corresponding to a couple time/temperature sufficient to pasteurize LDL dispersions causes a gelation, whereas high-pressure treatment with a similar effect only provokes thickening. The aggregation interactions should certainly be of hydrophobic nature, and we have to prove it clearly.

We can observe that the impact of high pressure on LDL aggregation is modulated by pH, as at pH 3 such a phenomenon is not observed. At pH 3, it seems that high-pressure treatment does not denature apoproteins, which is the first step to LDL aggregation. We have observed in this paper (**Figure 3**) that pH 3 induced a first denaturation in nontreated LDL as compared to pH 8: a denaturation enthalpy of 1.3 mJ/mg of protein at pH 3 versus 5.5 mJ/mg of protein at pH 8. This signifies that proteins of LDL endure structural modification during acidification but that these modifications have no impact on LDL structure and subsequent LDL aggregation. However, these modifications are important as evidenced by the low value of the resulting denaturation enthalpy.

On the other hand, high-pressure treatment at 600 MPa at pH 8 provokes a decrease of denaturation enthalpy less pronounced than with pH but with a clear correlation with subsequent LDL aggregation. This obviously indicates that mechanisms of denaturation between pH and high pressure are different. It could be that protonation of side chains affects the structure of proteins in such a mode that they are then less sensitive to high-pressure treatment. Furthermore, as protein isoelectric points are situated in the range of 6.5−7.3 (*11*), we can suggest that protonation leads to more repulsive interactions limiting subsequent hydrophobic interactions for aggregation. Thus, pH changes certainly modify the balance between electrostatic and hydrophobic interactions leading to a modified reactivity toward high pressure.

Techniques such as dynamic fluorescence spectroscopy could give us some information about the impact of pH and high pressure on the structural changes of proteins embedded in LDL structure, as we can see in the domain of biological membranes (*27*).

HP Decreases Bridging and Depletion Flocculation at pH 8. We have seen in this paper that, at pH 8, severe high-pressure treatment (more than 400 MPa) modifies characteristics of LDL protein denaturation and of LDL aggregation and that these changes provoke a decrease of flocculation in emulsion made with the treated LDL samples.

Recently (*28*), we have evidenced that previous high-pressure treatment of soy protein isolates altered their subsequent emulsifying properties and particularly decreased bridging flocculation. However, in this case, we treated soluble proteins without lipids, whereas in the present study, samples are supramolecular assemblies constituted of a core of neutral lipids surrounded by a film of proteins and phospholipids. We have recently demonstrated that LDL assembly renders possible the transport of emulsifier molecules (phospholipids and highly hydrophobic proteins) that could not be soluble and available to travel to the oil−water interface (*5*). Then at the interface, LDL spread and the emulsifiers adsorb. We have also evidenced that the protein part is essential in the initial anchorage of the assembly (*29*).

In the present study, we noticed that both depletion and bridging flocculation are reduced when using high-pressure treated LDL at pH 8. First, we can remark that the nontreated LDL dispersion at pH 8 presented a high depletion flocculation level and that the treatment permits the drastic diminishment of this level. On the other hand, at pH 3, conditions that allowed another form of protein denaturation but without aggregation, we have noticed that the depletion flocculation level was very low whatever the high-pressure treatment. It appears that the denaturation/aggregation of LDL at pH 8 does not alter the capacity of protein adsorption as we have checked the same percent of adsorbed proteins and the same size of droplets than with nontreated samples. We could suppose that with sufficient high-pressure treatment, nonadsorbed LDL can interact with water through the polar side chains of proteins and the polar head of phospholipids. The subsequent treatment at 600 MPa could have modified these interactions, rendering the nonadsorbed LDL less interactive with water but without decreasing the solubility due to the low density caused by the high content of lipids in the supramolecular structure. Thus, the poor interaction with water could decrease the depletion effect.

Concerning the bridging flocculation, we have the same evolution as a function of pH and high pressure. The apparition of LDL aggregation for 600 MPa is correlated by a rough decrease in bridging flocculation. We could assess that the big aggregates formed during the 600 MPa treatment can adsorb to the surface of more than one colloidal particle, provoking potential bridging, but that the modified structure is not very strong and that it rapidly disrupts during homogenization. In the other conditions, the LDL spread at the interface and the nondenaturated LDL coming additionally to a covered interface could interact with adsorbed proteins forming a stable bridge between droplets, due to the robustness of the native nonadsorbed LDL structure. We have to assess this hypothesis by trying to visualize the aggregated LDL in solution and at an interface in situ using cryotomographic transmission microscopy.

CONCLUSIONS

High-pressure treatment of LDL dispersion brings some modifications of physicochemical characteristics and functionality mainly at pH 8. Particularly, aggregation and protein denaturation are enhanced, and the consequences of these modifications on LDL emulsifying properties are a significant decrease of depletion and bridging flocculation. However, these modifications do not influence the capacity of LDL adsorption at the oil−water interface as we have observed the same percentage of adsorbed proteins. In conclusion, a high-pressure

treatment of LDL dispersions combined with alkaline pH could lead to the fabrication of more stable oil−water emulsions.

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