JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Dry-Heating Makes Hen Egg White Lysozyme an Efficient Foaming Agent and Enables Its Bulk Aggregation

YANN DESFOUGÈRES,*,† VALÉRIE LECHEVALIER,† STÉPHANE PEZENNEC,† FRANCK ARTZNER,§ AND FRANÇOISE NAU†

UMR1253, Science et Technologie du Lait et de l'Oeuf, INRA, Agrocampus Rennes, 35000 Rennes, France, and UMR6251, Institut de Physique de Rennes, CNRS, Université Rennes 1, 263 Avenue du général Leclerc, 35042 Rennes cedex, France

Dry-heating is considered to be one of the most promising approaches to improvin g the functionality of food proteins. It has been shown that even if only minor structural modifications occur during dry-heating, the foaming properties of proteins are highly improved. With the recent results obtained in the field of foam stabilization by nanoparticles or protein aggregates in mind, a study was undertaken on the impact of dry-heating of lysozyme, used as a model protein, on its foaming properties. This work highlighted the fact that dry-heated hen egg white lysozyme simultaneously exhibited enhanced foaming properties and aggregation capacity. Although the conditions that favored bulk aggregation (high ionic strength, pH, treatment duration, and protein concentration) also favored foaming properties, the large bulk aggregates were not essential to obtain the best functionality. It is envisaged that heat-treated lysozyme may self-associate at the air/water interface, stabilizing air bubbles.

KEYWORDS: Dry-heating; foaming properties; hen egg white lysozyme; aggregate

INTRODUCTION

The mechanisms of formation and stabilization of foams are of particular interest for food manufacturers. Foams are thermodynamically unstable systems because of drainage, coalescence, and disproportionation. Proteins are the most widely used stabilizers in the food industry, but they often cannot provide sufficient long-term foam stability. Different methods have been developed to improve the functionality of the proteins: heat treatment in solution (*1*), high-pressure treatment (*2*), glycation by the Maillard reaction (*3*), succinylation (*4*), phosphorylation (*5*), and dry-heating (DH) (*6*). The last one seems to be the most promising approach. First, it has been shown to improve the foaming properties of proteins without a significant loss of solubility. Second, it is a process that can be used easily in an industrial context and provides ready-to-use food powders. Finally, the treatment can be combined with glycation and phosphorylation when the DH of protein powders is performed in the presence of polysaccharides or pyrophosphate, respectively (*7–9*). Furthermore, in the case of hen egg white powder, DH eliminates microbial contaminations because it was first developed with this aim. However, the structural modifications of the proteins induced by such a treatment responsible for the improvement of the foaming properties are not fully elucidated.

Hen egg white (HEW) is extensively used in the food industry due to its nutritional and functional properties. Kato et al. (*10*) have demonstrated that a 7-day dry-heating treatment (80 °C, 7.5% moisture content) was responsible for the best foaming properties, and further studies showed that the treatment induced only mild structural modifications and the formation of soluble aggregates (*10, 11*). Mine (*12, 13*) and Van der Plancken et al. (*2*) pointed out that the extent of modifications induced by dry-heating was related to the buffer pH used during spray-drying and the moisture content of the resulting powder. These modifications partly identified by Matsudomi et al. (*11*) in the case of the DH of ovalbumin, the major protein of HEW, have not been directly linked to the interfacial activity of the proteins modified by this treatment.

In the case of the thermal treatment of protein solutions, the formation of soluble and/or insoluble aggregates has been shown to favor the increase of foaming properties (*14, 15*). The formation of these aggregates depends highly on the pH and ionic strength and may result from the unfolding of the polypeptide chains. Relkin et al. (*16*) showed that a molten-globule-like state of ovalbumin can be obtained from a mild heat treatment, which is responsible for the improvement of the foaming functionality, probably due to the enhanced flexibility and surface hydrophobicity.

HEW is a complex mixture of many proteins and other organic and inorganic compounds (carbohydrates, minerals,...). The use of such a system to study the link between molecular structure and functional properties is difficult. Obtaining infor-

* Corresponding author (telephone +33 223485575; e-mail yann.desfougeres@agrocampus-rennes.fr).
† INRA.
§ CNRS.

10.1021/jf703715j CCC: $40.75
Published on Web 06/10/2008

mation about the impact of physicochemical treatments on protein structure and functional properties often requires simplifying the system chosen. In this work, we used hen egg white lysozyme (HEWL) as a model protein. HEWL has been used as a model for a wide range of studies including air/water interfacial behavior (*17–21*). It is a globular protein composed of 129 amino acids and contains 4 disulfide bridges, which reduce the flexibility of the polypeptide chain. This characteristic combined with a low surface hydrophobicity makes lysozyme a very poor foaming agent. The aim of this study was to assess whether or not DH had a significant effect on the foaming properties of lysozyme with the fact that this protein has a very rigid structure and does not have a propensity to foam in its native state in mind. Therefore, we determined the foaming properties of dry-heated hen egg white lysozyme (DHEWL) and tried to propose a mechanism to explain the modified functionality.

MATERIALS AND METHODS

Dry-Heating of Lysozyme. Spray-dried hydrochloric lysozyme was supplied by Ovonor (Annezin-les-Béthune, France). The pH of a 10 mg mL^{-1} solution of lysozyme in ultrapure water is 3.6. The powder (moisture content = 7%, water activity, $a_w = 0.31 \pm 0.02$) was placed in hermetically capped glass tubes and stored in an oven at 80 °C for 0−7 days. At the end of the treatment, all samples were kept at 4 °C.

Measurement of Solubility. The protein samples were dissolved at a concentration of 10 mg mL^{-1} in 10 mM sodium phosphate buffer, pH 7.0, before centrifugation (10000*g*, 15 min). The protein concentration in the initial solution and in the supernatant was determined according to the method of Lowry et al. (*22*).

Foaming Properties. Foaming properties were analyzed as described by Baniel et al. (*23*) using a bubbling column PM930 (Grosseron, St. Herblain, France). Compressed air was injected at a constant flow rate (25 mL min^{-1}) in 12 mL of the sample through a porous metallic disk placed at the bottom of the column. Bubbling was stopped when the foam volume reached 50 mL (*V*). The evolution of the foam was then recorded for 30 min. Conductivity measurements were used to calculate the volume of liquid in the foam and thus allowed the foaming capacity (FC), the foam density (FD), and the long-term stability (FS) to be characterized. These parameters were calculated with eqs 1, 2, and 3, respectively:

$$FC = V_a/V \quad (1)$$

$$FD = V_m/V \quad (2)$$

$$FS = V_f/V_m \times 100 \quad (3)$$

V_a is the volume of air necessary to produce 50 mL of foam, V_m is the maximum volume of liquid incorporated in the foam, and V_f is the volume of liquid remaining in the foam after 30 min.

The short-term stability or flow drainage rate (FDR, mL s^{-1}) was estimated by the slope of the curve "liquid volume in the foam versus time", just after the end of bubbling. The lower the FDR observed, the better the short-term stability.

The protein solutions (10 mg mL^{-1}) were prepared either in a 23 mM ionic strength buffer (10 mM sodium phosphate, pH 7.0) or in 140 mM ionic strength buffers (60 mM sodium phosphate, pH 7.0, or 34.4 mM sodium citrate, 32.2 mM sodium phosphate, pH 3.6, or 50 mM Tris-HCl, 132 mM NaCl, pH 9.0).

When needed, lysozyme aggregates were eliminated by filtration through 0.20 μm filters (Sartorius AG, Goettingen, Germany) 24 h after solubilization of lysozyme at 15 mg mL^{-1} in 60 mM sodium phosphate, pH 7.0. The concentration of the filtrate was then adjusted spectrophotometrically at 280 nm to 10 mg mL^{-1} using the molar extinction coefficient 37750 M^{-1} cm^{-1} (*24*). A Zetasizer 3000HS (Malvern Instruments, Malvern, U.K.) was used to verify the absence of aggregates by dynamic light scattering (size range = 1.5 nm−3 μm). The instrument used a 10 mW helium−neon laser at 632.8 nm to illuminate the samples, and the scattered light was collected at 90°. A correlator function was used by the software of the instrument to derive the particle size.

Table 1. Aggregation Parameters of Native and DHEWL (7 Days) at pH 3.6, 7.0, and 9.0 at Room Temperature[a]

	aggregation parameters	
sample	initial aggregation rate ($\times 10^{-3}$ absorbance unit per min)	A_{600nm} (240 min after dissolution)
native lysozyme, buffer pH 3.6	0	0
native lysozyme, buffer pH 7.0	0	0.01
native lysozyme, buffer pH 9.0	0	0.01
DHEWL, buffer pH 3.6	0	0
DHEWL, buffer pH 7.0	9.57 ± 0.52	0.89 ± 0.03
DHEWL, buffer pH 9.0	11.18 ± 0.2	0.82 ± 0.01

[a] All of the buffers were prepared for a constant ionic strength of 140 mM. pH 7.0 buffer was 60 mM phosphate; pH 3.6 buffer was 34 mM citrate/32.2 mM phosphate, 85 mM NaCl; pH 9.0 buffer was 50 mM Tris-HCl, 132 mM NaCl. The protein concentration was 10 mg mL^{-1}.

Aggregation Kinetics. For the determination of the aggregation kinetics, turbidity measurements were performed on a Jenway 6505 UV−vis spectrophotometer (Jenway, Chelles, France) at 600 nm during 400 min. The protein samples were prepared at concentrations varying from 2.5 to 10 mg mL^{-1} in the buffers described above and in 60 mM sodium phosphate, 2% Triton X-100, pH 7.0. The experiments were conducted at room temperature or at 4 °C. The initial rate of aggregation was defined as the value of the slope of the curve "A_{600nm} versus time" when aggregation started.

Aggregate Stability. Aggregates were prepared by dissolving 10 mg of lysozyme in 1 mL of 60 mM sodium phosphate, pH 7.0. The samples were left for 24 h at room temperature. The protein solutions were then centrifuged (10000*g*, 30 min) to isolate the aggregates. One milliliter of different destabilization buffer was added to the pellet, and the samples were left at room temperature for 30 min under agitation. The turbidity of the solution was measured at 600 nm as described above. The compositions of the destabilization buffers are given in **Table 2**. Then, dithiothreitol (DTT) was added to each tube as a reducing agent (final concentration = 0.1 M) and the absorbance was measured again at 600 nm, 5 min after the addition.

Dark-Field Optical Microscopy. Lysozyme solutions were prepared at 10 mg mL^{-1} in 60 mM sodium phosphate buffer, pH 7.0. Samples were observed with an Olympus IX70 microscope (Olympus, Rungis, France) equipped with a dark-field condenser (N.A. 0.8−0.9) (*25*). Images of the scattered light were recorded with an Olympus Camedia 4040 photo apparatus.

Statistics. All of the experiments were performed in triplicate. On the figures, the bars represent the standard deviation. When needed, the numerical data were statistically analyzed using Statgraphics Plus 5.1 software. For each parameter (FC, FD, FDR, and FS), the values were analyzed using a Student *t* test at the $p < 0.001$ (99.9% confidence) level.

RESULTS

Dry-Heating Enhances Foaming Properties of Lysozyme without Loss of Solubility. No loss of solubility was observed during the 7 days of treatment when the protein samples were prepared in 10 mM sodium phosphate buffer, pH 7.0 (data not shown). Dry-heating at 80 °C improved drastically the foaming properties of lysozyme dissolved in 60 mM sodium phosphate buffer, pH 7.0. Although the foaming capacity did not change over the period of heating, the foam density, foam stability, and foam drainage rate reached 189, 525, and 7% of the values obtained for each parameter with native lysozyme, respectively (**Figure 1**). To obtain the maximum change, 0.5 day was sufficient for FD and 2 days for FDR and long-term stability.

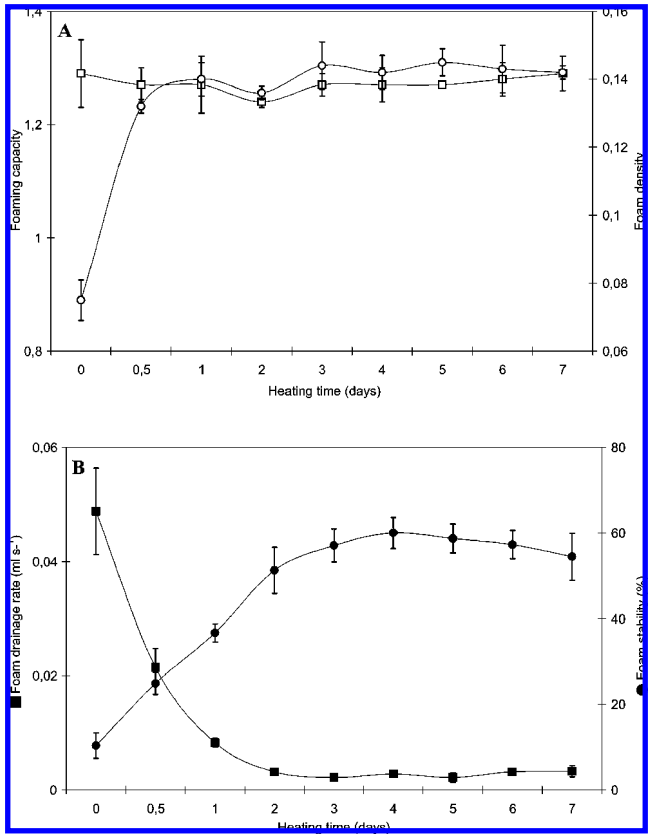

Figure 1. Foaming properties of hen egg white lysozyme during dry-heating: (**A**) foaming capacity (□) and foam density (○); (**B**) foam drainage rate (■) and long-term stability (●). After heat treatment, the protein solutions (10 mg mL^{-1}) were prepared in 60 mM phosphate buffer, pH 7.0. The time range of the long-term stability was 30 min.

For a longer time of heating, the foaming properties did not change significantly.

The buffer ionic strength increase did not affect the FC of native lysozyme or DHEWL after 7 days (**Figure 2**). For native lysozyme, an increase of the ionic strength induced the formation of a denser (higher FD) but less stable (lower FDR) foam. Considering FS, no conclusion could be drawn for native lysozyme. Indeed, with this protein solution, the volume of liquid remaining in the foam at the end of the trial (V_f) was constant whatever the experimental conditions because of the very poor foaming properties of native lysozyme. Actually, V_f was very low and may correspond to the volume of foam adsorbed on the edge of the column. Finally, with native lysozyme solutions, the final volume V_f is a constant and the FS value is then simply inversely proportional to the FD value, considering eqs 2 and 3 under Materials and Methods. For DHEWL, an increase of the ionic strength induced the formation of a less dense (lower FD) but more stable (lower FDR and higher FS) foam. Thus, by comparison of native lysozyme and DHEWL, it is interesting to note that an increase in ionic strength has an opposite effect on FD and FDR.

The foaming properties of native lysozyme were so weak that it was not possible to produce 50 mL of foam at pH 3.6 (**Figure 3**); then it was not possible to measure any foam parameter. Moreover, as described above, no residual foam remained at the end of the experiment when native lysozyme was used, at pH 7.0 as well as at pH 9.0; FS was then not considered for these samples. With native lysozyme, FC, FD, and FDR decreased when the pH increased from 7.0 to 9.0. The same trend was observed with DHEWL for FC and FD, but no significant difference was observed for FDR and FS. The foam obtained at pH 3.6 displayed lower FD and FS and a higher FDR than the foam produced at pH 7.0, whereas no difference was observed for FC. Whatever pH conditions (from 3.6 to 9.0), DHEWL exhibits improved foaming properties compared to native lysozyme.

Dry-Heating Induces Bulk Aggregation of Hen Egg White Lysozyme. An aggregation process was observed after dissolving dry-heated lysozyme in 60 mM sodium phosphate buffer, pH 7.0 (**Figure 4**). This phenomenon began abruptly as soon as the protein was dissolved, without a lag phase. After 400 min, the aggregation reached a more stable state; the rate of aggregation decreased dramatically even if a continual increase of the turbidity was observed during the course of the experiment (24 h). As shown by dark-field optical microscopy, micrometer size particles coagulate to form aggregates that are not well-defined and are heterogeneous in size (**Figure 5**).

The rate of aggregation increased exponentially with the increase of dry-heating time (**Figure 4A**). Moreover, the higher the protein concentration was used, the higher the aggregation rate (**Figure 4B**). At low ionic strength, that is, below 35 mM, no aggregation was observed (**Figure 4C**). Then, the aggregation rate increased while the ionic strength was increased up to 105 mM. Beyond this ionic strength, the rate did not increase further. **Table 1** shows that native lysozyme did not aggregate at any pH between 3.6 and 9.0. However, for DHEWL, the aggregation was pH-dependent: no aggregates were detectable at pH 3.6, in contrast with pH 7.0 and pH 9.0, and the aggregation rate increased when the pH was increased from 7.0 to 9.0. It is, however, noticeable that the optical density after 4 h was higher at pH 7.0 compared to pH 9.0. Actually, at pH 9.0, a plateau was reached 2 h after protein dissolution. These observations could be related to different aggregate sizes depending on the pH, because the optical density is a simultaneous measurement of aggregate concentration and size. This assumption agrees with the big particulates, which could be distinguished by the bare eye and which rapidly appeared at pH 9.0, which was not the case at pH 7.0.

To characterize the type of interactions involved in the aggregation process, we tried to destabilize the preformed structures with different detergents and a chaotropic agent. **Table 2** shows that the aggregation was irreversible upon dilution or ionic strength decrease. A neutral non-denaturing detergent such as Triton X-100 induced a reduction of only 15% of the aggregates. However, practically all of the aggregates disappeared when resuspended in 2% SDS or 8 M urea and about 25% disappeared when resuspended in pH 3.6 buffer. The addition of DTT improved the aggregate destruction with SDS and urea and at low pH, but did not significantly modify the aggregates stability in the other conditions. The slight decrease observed after its addition in the other cases may result from the dilution of the samples. Moreover, the aggregation rate was drastically decreased when the temperature was lowered to 4 °C (pH 7.0, μ = 140 mM) (**Table 3**). The addition of 2% Triton X-100 also decreased the aggregation rate, but the phenomenon was not fully abolished. The final value of the absorbance (600 nm) is even higher than in the absence of the surfactant. This result suggests that Triton X-100 may limit the size but not the number of the aggregates, because the optical density may be higher for a great number of small aggregates than for a small number of big ones.

Foaming Properties Increase Is Not Solely Due to Large Bulk Aggregates. To determine if the large aggregates could be responsible for the increase of the foaming properties, aggregates were prepared using a solution of DHEWL (7 days)

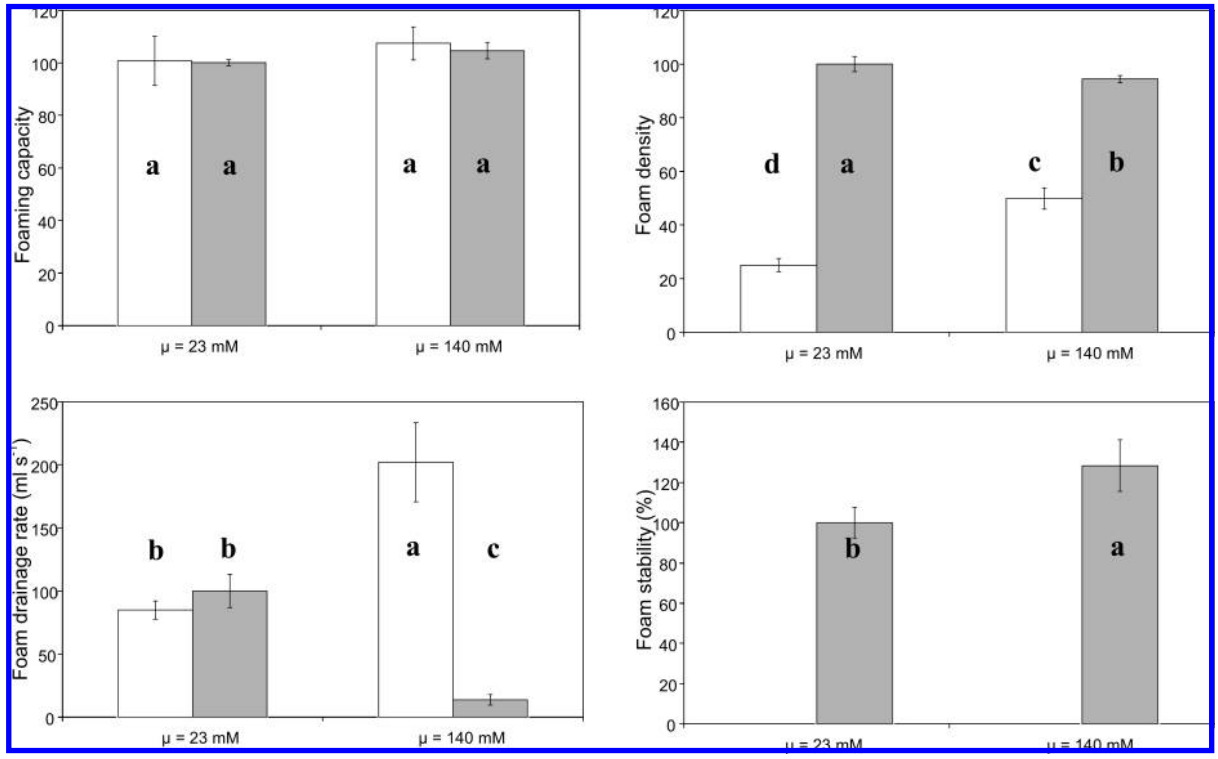

Figure 2. Foaming properties of native lysozyme (white bars) and dry-heated lysozyme for 7 days (gray bars) solubilized in 10 mM (μ = 23 mM) or 60 mM (μ = 140 mM) phosphate buffer, pH 7.0. The results are expressed as percentages of the values obtained for the DHEWL at μ = 23 mM. For each parameter, significant differences are indicated by different letters (Student *t* test, $p < 0.001$).

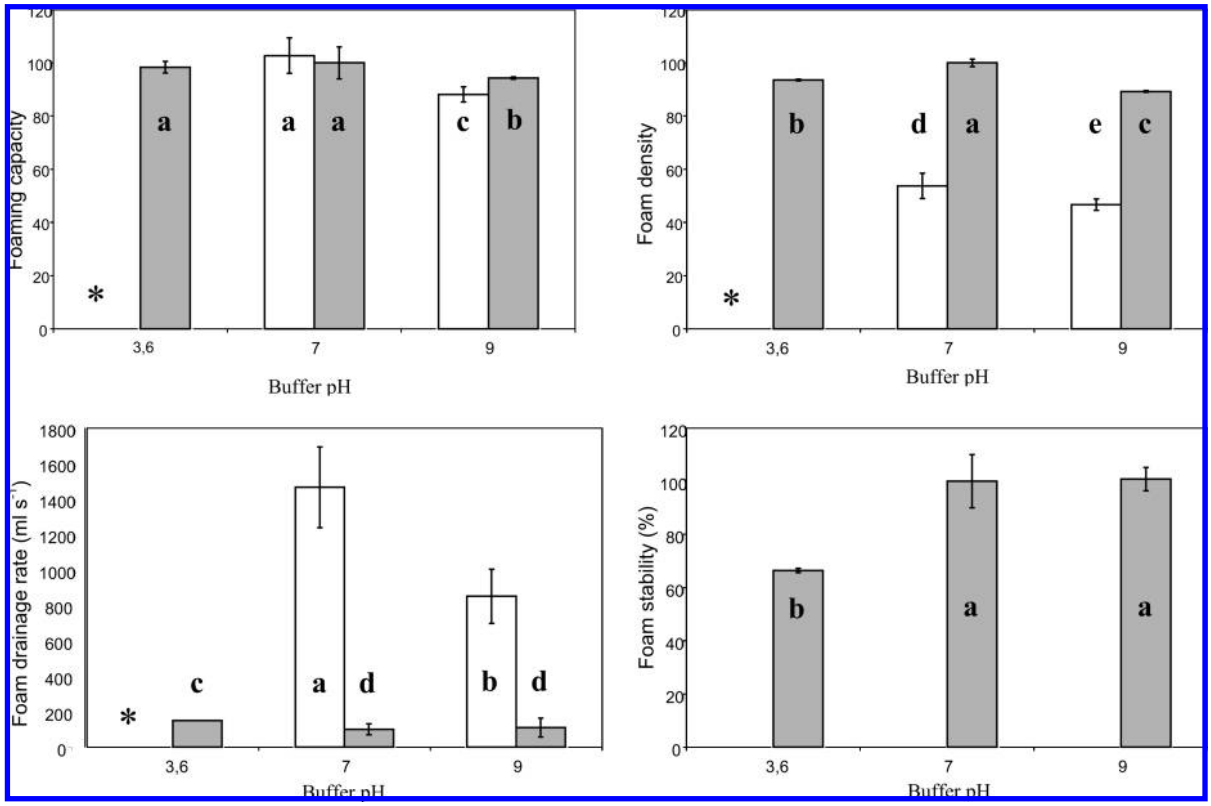

Figure 3. Foaming properties of native lysozyme (white bars) and lysozyme dry-heated for 7 days (gray bars) solubilized in 34.4 mM citrate/32.2 mM phosphate buffer, 85 mM NaCl, pH 3.6, 60 mM phosphate buffer, pH 7.0, or 50 mM Tris-HCl buffer, 132 mM NaCl, pH 9.0. In each case μ = 140 mM. ∗, no foam could be produced at pH 3.6 with native lysozyme. The results are expressed as percentages of the values obtained for the DHEWL in the pH 7.0 buffer. For each parameter, significant differences are indicated by different letters (Student *t* test, $p < 0.001$).

at 15 mg mL^{-1} in 60 mM phosphate buffer, pH 7.0, and kept at room temperature for 24 h. After filtration (0.20 μm), the supernatant concentration was adjusted to 10 mg mL^{-1}. The absence of aggregates in the solution was verified by dynamic light scattering. Before filtration, very large aggregates could be observed, but their size could not be determined because it

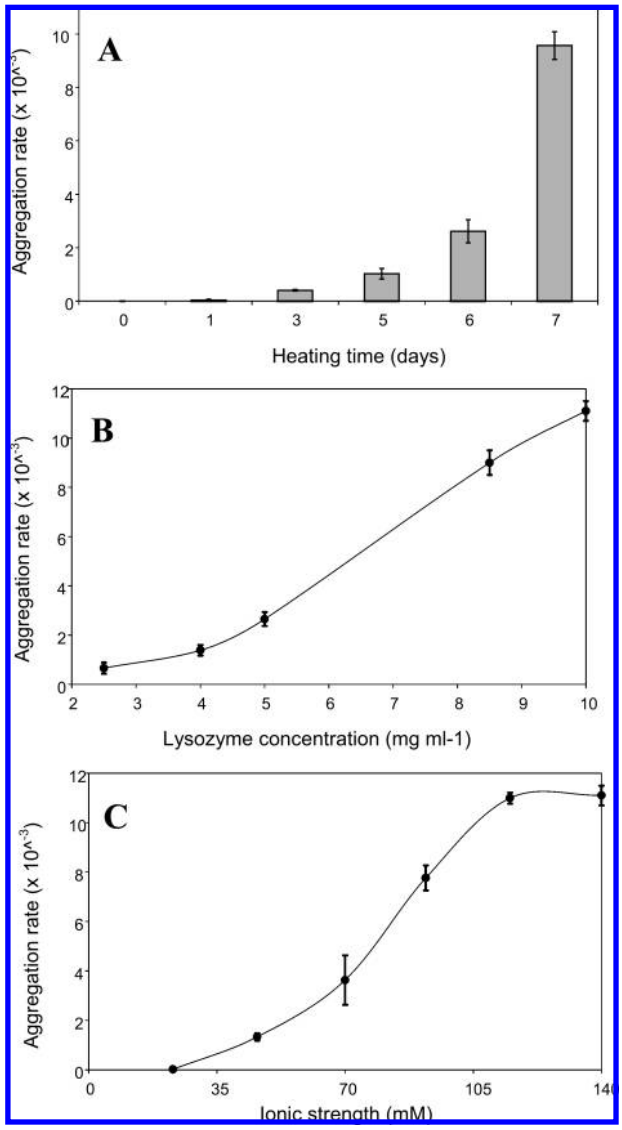

Figure 4. Dependence of the aggregation rate on (**A**) dry-heating time, 10 mg mL^{-1} lysozyme in 60 mM phosphate buffer, pH 7.0, (**B**) protein concentration, 7 days DHEWL in 60 mM phosphate buffer, pH 7.0, and (**C**) ionic strength, 7 days DHEWL at 10 mg mL^{-1}. The aggregation rate is expressed as the increase of the absorbance at 600 nm per minute when the aggregation began.

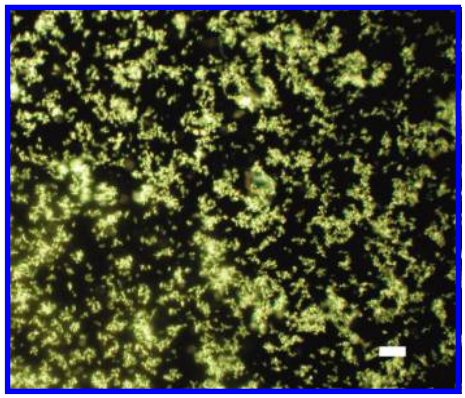

Figure 5. DHEWL aggregates observed by optical microscopy (dark field). Seven-day-dry-heated lysozyme was solubilized in 60 mM phosphate buffer, pH 7.0, at 10 mg mL^{-1}. The white bar represents 50 μm.

was out of the size range of the instrument. After filtration and during the following 3 h, a single signal identical to the one

Table 2. Stability of the Aggregates toward Different Detergents and Denaturants[a]

nature of the dissolution buffer	percentage of residual turbidity (A_{600nm}) − DTT 0.1 M	+ DTT 0.1 M
60 mM phosphate buffer, pH 7.0	100.7 ± 1.8	98.9 ± 0.6
deionized water	95.5 ± 1.1	92.7 ± 1.3
60 mM phosphate buffer, pH 7.0, 2% Triton X-100	86.4 ± 1.9	89.9 ± 2.7
34 mM citrate/32.2 mM phosphate buffer, pH 3.6, 85 mM NaCl (μ = 140 mM)	73.8 ± 2.2	68.5 ± 2.3
60 mM phosphate buffer, pH 7.0, SDS 2%	4.2 ± 0.0	0.8 ± 0.0
60 mM phosphate buffer, pH 7.0, 8 M urea	1 ± 0.0	0 ± 0.0

[a] One milliliter samples of DHEWL (7 days) were prepared in 60 mM phosphate buffer, pH 7.0, at 10 mg mL^{-1} and left at room temperature for 24 h. The aggregates were then isolated by centrifugation (10000*g*, 30 min) and resolubilized in 1 mL of the same buffer containing various detergents or denaturants. The percentage represents the residual turbidity at 600 nm.

Table 3. Aggregation Parameters of DHEWL (7 Days) in 60 mM Phosphate Buffer, pH 7.0, at Room Temperature and at 4 °C and in the Presence of 2% Triton X-100

sample	aggregation parameters: initial aggregation rate ($\times 10^{-3}$ absorbance unit per min)	aggregation parameters: A_{600nm} (240 min after dissolution)
DHEWL, buffer pH 7.0, 20 °C	9.57 ± 0.52	0.89 ± 0.03
DHEWL, buffer pH 7.0, 4 °C	0.71 ± 0.09	0.22 ± 0.04
DHEWL, buffer pH 7.0, 2% Triton X-100, 20 °C	7.05 ± 0.07	1.07 ± 0.01

obtained with native lysozyme was observed. However, the value of the diameter measured is focused on 3.9 nm for both the samples (native and dry-heated), whereas the literature indicates that HEWL diameter is 3.2 nm (*26*). The difference observed may be due to the detection limit of the apparatus. The presence of small oligomers (dimers or trimers) could not be excluded. Foams were immediately prepared with this aggregate-free sample, and the foaming properties were compared to those of a sample still containing aggregates (**Figure 6**). Neither FC, nor FD, nor short- and long-term stabilities were significantly affected by the presence of the aggregates. The high value of the standard deviation for the FDR is due to the difficulty to determine the slope of the curve at the very beginning of the destabilization process.

DISCUSSION

Dry-Heating Enhances Foaming Properties of Lysozyme. As shown earlier for the whole hen egg white (*6*) or purified proteins such as ovalbumin, ovotransferrin, and lysozyme (*10*), dry-heating importantly improves the functionality of the proteins. The effect was more striking with lysozyme because this protein has very poor foaming properties in the native state (*27*) due to its low surface hydrophobicity, its low flexibility, and its high surface net charge at pH below 8.0. For the whole hen egg white, Kato et al. (*6*) reported that the optimal functional properties were obtained after 10 days of dry-heating at 80 °C of a 7.5% moisture content powder. In the present study, only 2 days of treatment was sufficient to obtain the best foaming properties of lysozyme: the highest foam density and stability and the lowest foam drainage rate. Foaming capacity represents the rate of foam formation at a given air injection flow rate. This parameter did not change during heating or when the ionic

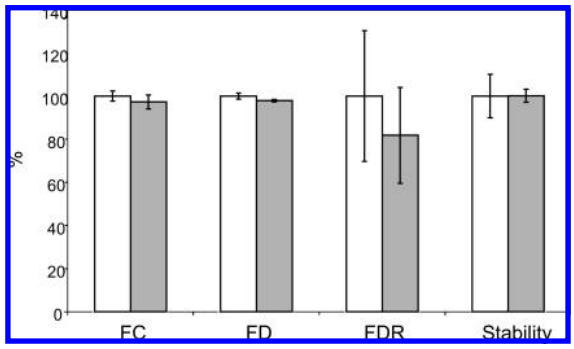

Figure 6. Foaming properties of DHEWL solutions containing aggregates (white bars) and without aggregates (gray bars). The foams were prepared at a protein concentration of 10 mg mL^{-1} in 60 mM phosphate buffer, pH 7.0. The results are expressed as percentages of the value obtained in the presence of the aggregates.

strength of the reconstituted lysozyme solution was modified, probably because of the high protein concentration used for foam formation (10 mg mL^{-1}), which could mask the effect of the protein modifications. However, it was not possible to use lower protein concentrations. Indeed, native lysozyme has so low a propensity to foam that it was not possible to determine any foaming parameters at protein concentrations below 10 mg mL^{-1}. However, this was essential for the estimation of the effect of DH on the foaming properties, and that explains the choice of this lysozyme concentration used throughout this work.

Ionic Strength Has an Opposite Effect on Foaming Properties of the Native and Dry-Heated Lysozyme. For the native lysozyme, increasing the ionic strength induced an increase of the foam density and a decrease of the short-term stability. A possible explanation is that the screening of surface charges of lysozyme allows a more rapid and increased adsorption at the air/water interface because of the decrease in electrostatic repulsions. However, the native structure of the protein does not enable sufficient intermolecular interactions to create a strong cohesive film stabilizing the foam. Such an interfacial film is generally the result of proteins forming an elastic network with high surface pressure (*28*). In the literature, the increase of the ionic strength either improves (*29*) or decreases (*30*) the foaming capacity of proteins. Townsend and Nakaï (*27*) showed that the foaming capacity of ovalbumin was 2.5 times higher when the ionic strength increased from 0.01 to 0.2 M; however, no change was noticed for lysozyme in these conditions.

The increase in ionic strength induced no change of the DHEWL FC but a slight decrease of the FD. For FC, the high protein concentration used may mask the effect of the environmental modifications (ionic strength and pH) as already mentioned above. For FD, the difference observed between the native and dry-heated samples can be explained by a decrease of the electrostatic repulsions induced by dry-heating, which favors hydrophobic interactions at the air/water interface. The short- and long-term stabilities of the foam produced with DHEWL were enhanced at high ionic strength. This result suggests that once the electrostatic repulsions are abolished, intermolecular interactions can occur between the denatured proteins, favoring the formation of a cohesive film at the air/water interface. This kind of interaction may be possible because of an exposure of reactive patches of the molecule due to the dry-heating and the possible induced structure modifications.

Dry-Heating Allows Lysozyme to Foam at Any pH. The foaming capacity and foam stability of many purified proteins such as ovalbumin and lysozyme (*27*) increased when the foams were prepared at a pH near the isolectric point of the proteins. It has been shown that the rate of adsorption and the amount of protein at the interface were higher when the protein carried no net charge, that is, at its isoelectric point (*31*). Moreover, surface rheological studies of protein films at the air/water interface indicated that a more rigid film was formed at this pH (*32–34*). In the present study, no foam could be produced with native lysozyme when the experiment was performed at a pH (3.6) far from the p*I* (10.7). This was probably due to strong electrostatic repulsions that limit the protein adsorption and inhibit the interactions between lysozyme molecules at the interface or to a higher net charge, which provides the protein molecule with higher affinity for the hydrophilic water environment than for the less hydrophilic air phase.

When the pH increased from 7.0 to 9.0, the foaming capacity and the foam density decreased. This can be explained by a loss of solubility of the protein near the p*I* at high ionic strength, which can be unfavorable to the adsorption of the protein at the interface. This phenomenon has already been reported for other proteins such as caseins for which the foaming properties are related to their solubility (*35*), which is lowest at their p*I*. Patino et al. (*36*) and Ruiz-Henestrosa et al. (*37*) showed that the adsorption of soy globulins at the air/water interface decreased drastically close to the isoelectric point of the proteins and that the foaming was zero at this pH. This coincided with an aggregation behavior of these slightly unfolded soy globulins in the bulk and at the interface in the same conditions. In this study, aggregation was observed at pH 7.0 and 9.0. However, the rate of aggregation was higher at pH 9.0. This could be responsible for the decrease of foaming properties as seems to be the case for the soy globulins. Because the stability parameters increase with an increase of the pH from 7.0 to 9.0, one can suppose that it could be the result of a decrease of the net charge of lysozyme as stated by MacRitchie et al. (*31*), allowing stronger/closer interactions between lysozyme molecules adsorbed at the air/water interface. It is noticeable that the effect of the pH increase was significant even at high ionic strength (140 mM), suggesting that in these conditions electrostatic repulsions were still effective.

For dry-heated lysozyme, foams could be produced at pH 3.6, indicating that the heat treatment favors the protein adsorption and/or the interface film stability. From pH 7.0 to 9.0, the foaming capacity and the foam density decreased, probably for the same reasons as for the native protein. No difference could be observed for the short- and long-term stabilities of the foam produced at neutral pH and higher. This suggests either that the electrostatic repulsions which inhibit the protein interactions at pH 7.0 between native molecules are reduced after dry-heating, allowing the DHEWL to form a more cohesive film at the air/water interface, or that new molecular attractions appeared, strong enough to abolish the electrostatic repulsions independently of the pH value, between 7.0 and 9.0. However, the electrostatic repulsions are much higher at pH 3.6 and probably not completely offset by the attractions because the foam stability is significantly lower in these pH conditions.

It should be interesting to perform the experiment at lysozyme p*I*. At such a pH we should observe the higher foam stability because of the absence of electrostatic repulsions. However, the experiments were not performed at pH 10.7, the isoelectric point of lysozyme, because it was a pH value too high to be sure that it could not induce additional modifications.

Protein Aggregation Is Mainly Driven by Hydrophobic Interactions. The other striking point of this study is that dry-heated lysozyme spontaneously aggregated after solubilization,

the rate and the extent of the aggregation depending on the treatment duration, protein concentration, and ionic strength. These aggregates could be destabilized only when resuspended in the presence of SDS or high concentrations of urea or by decreasing the pH from 7.0 to 3.6. The addition of DTT only acts when proteins are previously unfolded by the detergent or the chaotropic agent and further decreased the stability of the aggregates. Neither a dilution nor a decrease in ionic strength permitted the destruction of these structures, whereas the addition of Triton X-100 destabilized 15% of them. Moreover, the aggregation rate was lowered when the temperature was lowered to 4 °C, that is, when hydrophobic interactions are reduced. Together, these results indicate that aggregation is an irreversible phenomenon mainly driven by hydrophobic interactions, whereas some disulfide bridges seem to be established between lysozyme molecules, but disulfide bond formation needs a previous disulfide bridge reduction, because lysozyme has no free thiol groups. Then, we have to suppose that dry-heating induced disulfide bond reduction that led to thiol/disulfide exchange and the formation of intermolecular disulfide bond, but this assumption could not be experimentally proved.

Large Bulk Aggregates Are Not Essential for the Improvement of Foaming Properties. The question arose as to whether these aggregates are involved in the enhanced foaming properties of dry-heated lysozyme compared to the native one. Indeed, the literature provides some examples of the positive effects of aggregates on the foaming properties of protein solutions. Zhu and Damodaran (*1*) demonstrated that it was possible to enhance either the foamability or the foam stability controlling the monomeric to polymeric whey protein ratio. The same effect was observed by Unterhaslberger et al. (*14*) with β-lactoglobulin-enriched WPI and by Sorgentini et al. (*38*) and Rawel and Muschiolik (*39*) with soluble aggregates of soy proteins. Those superstructures could increase the stability of the foam by increasing the viscosity of the solution, limiting the drainage effect. They may also have different physicochemical properties such as a higher surface hydrophobicity, which could favor their adsorption at the air/water interface. In the present study, the elimination of lysozyme aggregates did not significantly modify the foaming properties. Moreover, at low ionic strength (23 mM) or acidic pH (3.6) no aggregation could be measured. However, under these conditions the foaming properties of DHEWL were much better than those of native lysozyme. Furthermore, FD and the two stability parameters were largely improved after 1 day of DH, whereas no aggregation was observed. These results indicate that the presence of bulk aggregates in solution was not essential for the improvement of the foaming properties. However, the conditions that favor aggregation also favor the formation of stable foams. Therefore, one can suppose that the interactions involved in the aggregation process may be those acting in the foam stabilization.

Monomeric and/or Oligomeric DHEWL May Be Responsible for the Formation of a Cohesive Interfacial Film. As mentioned above, no significant aggregation could be observed for the shortest heating times (up to 1 day), whereas the foaming properties were already enhanced. We suppose that modified proteins are responsible for the improvement of foaming properties and that when these modified proteins reached a critical concentration (depending on the heating duration and, of course, the DHEWL concentration) in the solution, they start to aggregate. When the aggregates were discarded by filtration, the monomers and maybe small oligomers of DHEWL were still present in solution but at a concentration below the critical concentration, which did not allow the protein to aggregate but was sufficient to obtain improvement in foaming properties. When adsorbed at the air/water interface, the dry-heated denatured form of the protein turned out to be highly concentrated, because it is known that the protein concentration reached some hundreds of milligrams per milliliter in the interfacial layer. Lu et al. (*40*) showed that in the case of the adsorption of native lysozyme at a planar air/water interface, from a 1 mg mL^{-1} protein solution (pH 7.0, $\mu = 20$ mM), the monolayer thickness was 47 ± 3 Å for an area per molecule of 950 ± 50 $Å^2$. From these results, the estimated concentration of lysozyme in the interface was 2.5 ± 0.14 mg m^{-2} or 525 ± 29 mg mL^{-1}. Such concentrations are 50−300 times higher than the ones necessary to induce the aggregation process of lysozyme in a solution. Moreover, even if it is not possible to measure the ionic strength and the pH at the air/water interface, one can imagine that the sole presence of such a quantity of protein may drastically increase the ionic strength and modify the pH value. Under such conditions, we can hypothesize that dry-heated lysozyme molecules, either monomeric or oligomeric in the bulk, aggregate when they reach the interface as they would in the bulk because of local high concentration. In this study the nature of the most reactive species was considered to be either the monomeric form of lysozyme or small oligomers. The aim was to exclude a possible role of the larger aggregates in the improvement of the foaming properties. However, the existence or the absence of small oligomers still has to be determined as well as their surface activity.

Recently, a number of studies demonstrated that nanoparticles could have astonishing bubble-stabilizing properties (*41*). Above a certain diameter (around 10 nm) and for a contact angle with the aqueous phase of 90°, these particles can adsorb quasi-irreversibly to the air/water interface. Among proteins used to stabilize foams, only ovalbumin was found to have a similar power of stabilization. The reason is attributed to its ability for gelation at the air/water interface (*42, 43*). These results can be directly linked with observations made with nanoparticles. Indeed, Binks (*44*) noted that stable bubbles formed with polystyrene latex particles could be produced when the bulk concentration was close to the concentration of coagulation. Moreover, Dickinson et al. (*45*) showed that the efficiency for partially hydrophobic silica particles to stabilize bubble is dependent on a balance between aggregation in the bulk and adsorption at the air/water interface. The highest foam stability was obtained at such a particle concentration that a firm gel was observed in the aqueous phase contiguous to the adsorbed particle layer at the interface. In its native form, lysozyme is a compact protein with low hydrophobicity and an elevated positive net charge at pH 7.0 due to its high isoelectric point. These characteristics are the cause of the low propensity of native lysozyme to form foam, but dry-heating induces structural changes that seem to be responsible for an aggregation process in the bulk and the improvement of the foaming properties. However, this aggregated form of lysozyme molecules seems not to be responsible for the increase of the foaming properties, but the conditions that favored protein aggregation also favored the formation of stable foams. We hypothesize that DHEWL, the structure of which has been modified, is also able to aggregate at the surface of the bubbles to form a strong cohesive film that could be responsible for the remarkable increase in foam stability. The major interactions involved in this interfacial aggregation phenomenon should be hydrophobic interactions. Work is in progress to determine the effect of the treatment on the structure of lysozyme. On the other hand, we are studying the behavior of this protein at a planar air/water interface by

rheology measurements, ellipsometry experiments, and interfacial spectroscopy. In both cases, the roles of the monomers and the possible oligomers should be studied separately.

ABBREVIATIONS USED

HEW, hen egg white; DH, dry-heating; HEWL, hen egg white lysozyme; DHEWL, dry-heated hen egg white lysozyme; FC, foaming capacity; FD, foam density; FS, foam stability; FDR, foam drainage rate.

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Received for review December 20, 2007. Revised manuscript received February 22, 2008. Accepted April 16, 2008. This work was supported by the Conseil Régional de Bretagne.

JF703715J