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Stability of White Wine Proteins: Combined Effect of pH, Ionic Strength, and Temperature on Their Aggregation

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Supporting Information

ABSTRACT: Protein haze development in white wines is an unacceptable visual defect attributed to slow protein unfolding and aggregation. It is favored by wine exposure to excessive temperatures but can also develop in properly stored wines. In this study, the combined impact of pH (2.5−4.0), ionic strength (0.02−0.15 M), and temperature (25, 40, and 70 °C) on wine protein stability was investigated. The results showed three classes of proteins with low conformational stability involved in aggregation at room temperature: β-glucanases, chitinases, and some thaumatin-like protein isoforms (22−24 kDa). Unexpectedly, at 25 °C, maximum instability was observed at the lower pH, far from the protein isoelectric point. Increasing temperatures led to a shift of the maximum haze at higher pH. These different behaviors could be explained by the opposite impact of pH on intramolecular (conformational stability) and intermolecular (colloidal stability) electrostatic interactions. The present results highlight that wine pH and ionic strength play a determinant part in aggregation mechanisms, aggregate characteristics, and final haze.

KEYWORDS: *wine protein stability, haze formation, pH, ionic strength, temperature*

INTRODUCTION

The formation of protein haze in white wines is an important issue in enology. Such hazes can appear in bottled wines and represent an unacceptable visual defect. They are attributed to protein unfolding upon exposure to excessive temperatures during transport or storage, leading to their aggregation.[1−4] Instabilities may also develop in properly stored wines, according to very slow kinetics (>12 months).[5−7] Thus, stability tests and stabilization treatments are performed in the everyday winemaking practice to ensure the stability of the bottled products.[8] Stability is mostly assessed using heat tests, which do not necessarily reflect changes and aggregation phenomena liable to occur in real storage conditions. These tests are performed in conditions that induce the precipitation of most proteins (typically 60 °C for 6 h or 80 °C for 1 h), whereas the different protein classes exhibit different sensitivities with regard to heat-induced unfolding and precipitation.[4,9−11] Stabilization is achieved by bentonite fining (protein adsorption on clay particles). Although effective in preventing haze formation, this treatment is not selective enough and may adversely affect wine quality by inducing significant aroma loss and sometimes color alteration.[12] It also causes substantial volume loss and is a non-negligible source of waste.[13] More specific and reliable predictive tools, as well as stabilization processes alternative to bentonite fining, are thus needed. However, their development requires a better knowledge of the physicochemical mechanisms involved in haze development and of the impact of wine composition.

Four major protein classes have been identified in white wines: thaumatin-like proteins (18−24 kDa), chitinases (27−35 kDa), β-glucanases (37−41 kDa), and grape invertase (66 kDa).[5,10,11,14−16] Total protein concentrations are usually within the range of 15−300 mg L^{-1}.[17] Previous works have shown that haze formation is poorly correlated with total protein content. This is likely related to the different thermal stabilities of the different protein classes.[4,9−11] It is also attributed to the presence in wines of non-protein compounds that may prevent or trigger haze development: different works highlight the role played by polysaccharides, polyphenols, and ions such as sulfate in the heat-induced aggregation of wine proteins.[7,18−24] Despite recent progress, the exact impact of the wine matrix is not fully understood. Especially, the effect of wine pH and ionic strength has been only little studied despite the well-known impact of these two parameters on both protein conformational and colloidal stability.[25] Recent works highlight the part played by electrostatic interactions on the heat-induced aggregation of wine proteins and the need for further works on that subject.[9,19] A difficulty in enology is the time dependence of haze formation when temperatures on the order of 18−25 °C are considered. As hazes develop according to very slow kinetics, most of the studies dedicated to protein hazing have been conducted at elevated temperatures (60−80 °C). As a result, there is only little information concerning protein stability at ambient and intermediate temperatures. Such information is needed to propose more selective and effective stability tests and stabilization treatments.

The aim of the present work was to investigate the impact of the pH and ionic strength on the stability of white wine proteins, considering different temperatures: ambient temperature (25 °C), 40 °C (a temperature that can be reached during transport or storage), and 70 °C (a temperature corresponding to heat tests). At ambient temperature, concentrations higher than

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those usually encountered in wines were used to accelerate kinetics. Results obtained in real wines and in model systems were compared to check the impact of wine nonprotein compounds on stability.

MATERIALS AND METHODS

Wine. The Sauvignon white wine used in the present study was elaborated in 2009 at the Pech Rouge Experimental Unit (INRA, Gruissan, France). Following fermentation, the wine was cold stabilized to prevent the crystallization of tartaric salts and clarified. No bentonite fining was performed. After a final membrane filtration (0.45 μm), the wine was aliquoted in bottles and stored at 8 °C before use. Conventional enological parameters were analyzed according to Vine and Wine International Organisation methods. These parameters were as follows: ethanol, 11.5% v/v; pH, 3.2; total acidity, 4.9 g L^{-1} in H_2SO_4; total SO_2, 87 mg L^{-1}; free SO_2, 25 mg L^{-1}; total polyphenol index, 4.6. The wine ionic strength was evaluated from the concentrations of the main ions (K^+, 566 mg L^{-1}; Na^+, 12 mg L^{-1}; Ca^{2+}, 80 mg L^{-1}; Mg^{2+}, 70 mg L^{-1}; SO_4^{2-}, 39 mg L^{-1}) as being at least on the order of 0.02 M. Protein concentration in the Sauvignon wine was estimated at 160 mg/L. A heat stability test was performed (80 °C, 30 min). After cooling, the wine turbidity was 10 NTU: the wine was unstable.

Purification of Wine Proteins. Wine proteins were isolated and purified from the Sauvignon wine by ion exchange chromatography, using a cation exchange Streamline SP gel (74 mL, GE Healthcare) and a 350 mm length × 25 mm diameter column (GE Healthcare). The cation exchange phase was first swollen and equilibrated with a 13 mM tartrate sodium buffer (A) at pH 3.0 (flow rate = 9 mL min^{-1}, 1 h). The wine (3 L) was then loaded at a flow rate of 10 mL min^{-1}. Following 1 h of rinsing (buffer A, 10 mL min^{-1}, 1 h), the phase was packed. Bound proteins were eluted at 5 mL min^{-1} with a 13 mM tartrate buffer at pH 4.0 (buffer B) and the same buffer with added 0.5 M NaCl (buffer C) using the following gradient: 0−40 min, 100% B; 40−70 min, 100% B to 100% C; 70−80 min, 100% C. Protein elution was monitored by UV detection (280 and 320 nm). The protein-containing fractions were pooled, and salt removal was achieved by extensive diafiltration using buffer B. Diafiltration was performed in a 200 mL stirred cell with a 5 kDa membrane (Amicon, Millipore). Proteins in buffer B were concentrated to a final volume of 20 mL, corresponding to a protein concentration of 24 ± 1 g L^{-1}, and stored at −20 °C before use. Protein concentration in this stock solution was determined by lyophilization and weighing of 1 mL aliquots.

Protein Analysis and Identification. *1D Electrophoresis.* Protein analyses were performed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). Before analysis, protein concentration in the samples was adjusted to 0.8 g L^{-1}. This was achieved for wine by concentration using centrifugal filter units (3.0 kDa, Millipore), whereas the protein stock solution was diluted with water. Proteins in the Laemmli buffer were separated on a 14% acrylamide resolving gel (gel length = 60 mm). A low molecular weight calibration kit (Pharmacia, Biotech), ranging from 14.4 to 97 kDa, was included in each electrophoretic run. Gels were stained with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad) in 40% of ethanol and destained in 10% of acetic acid overnight. Gels were then scanned at 300 dpi with an image scanner (GE Biosciences). Image analysis was carried out with Totallab software (Nonlinear Dynamics Ltd.) and was used to calculate the proportion of proteins in each staining band.[11]

2D Electrophoresis. 2D electrophoresis was realized on the purified protein fraction according to the procedure described before.[11] Isoelectrofocusing (IEF) was carried out with 800 μg of protein using 18 cm long Immobiline Dry-strips (pH interval 3−10, nonlinear, GE Healthcare). Second-dimension SDS-PAGE was performed on a 12% acrylamide gel using an ISODALT apparatus (GE Healthcare). Gels were stained with colloidal CBB G-250 (Bio-Rad) and scanned.[26] Molecular weights and isoelectric points of spots were determined according to migration of 2D standards (Bio-Rad).

Identification of Proteins by Mass Spectrometry. Mass spectrometry analyses were done by the proteomic platform of the 1199 INRA Research Unit, on bands obtained from 1D electrophoresis gels.[27] The homemade *Vitis vinifera* database was queried locally using the Mascot search engine (v. 2.2.04; Matrix Science, London, U.K.) and with the following parameters: all entries for the taxonomy, trypsin as enzyme, one missed cleavage allowed, carbamidomethylation of cysteine as fixed modification, oxidation of methionine as variable modification, and 0.6 Da mass accuracy in both MS and MS/MS. Under these conditions, individual ion scores above 30 indicated identity or extensive homology ($p < 0.05$), and proteins were validated once they showed at least one peptide over this threshold.

Preparation of Model Solutions and Wine for Stability Experiments. Model solutions (12% ethanol, tartaric acid 2 g L^{-1}, and glycerol 7 g L^{-1}) were used to study protein stability at different pH values, ionic strengths, and temperatures. The pH was adjusted with HCl (1 M) or NaOH (1 M) and the ionic strength with NaCl. Model solutions were prepared with a concentration factor of 1.5 to obtain the required pH and ionic strength, followed by dilution with a protein solution at a given concentration. Model solutions were stored at 4 °C before use. Protein solutions (tartaric acid, 2 g L^{-1}, pH 4.0 adjusted with NaOH) were prepared by dilution of the stock solution to reach final concentrations in the samples of 8, 0.8, and 0.16 g L^{-1}. Final pH values were 2.5, 3.0, 3.2, 3.5, and 4.0 and final ionic strengths, 0.02 and 0.15 M.

To study stability at ambient temperature, protein concentration in the initial wine was increased using osmotic stress.[28] To this end, a 3.5 kDa cutoff dialysis bag (diameter = 29 mm, Spectra/Por) was filled with 50 mL of wine and immersed in a 125 g L^{-1} solution of polyethylene glycol (PEG, 35 kDa, Sigma) dissolved in protein-free wine. The protein-free wine was recovered following protein retention by cation exchange chromatography. The cutoff of the dialysis bag was chosen to allow only solvent, ions, and small molecules exchange between the two compartments, whereas wine polysaccharides and proteins were retained. The concentration factor (5) was determined by weighing. Wine pH was adjusted between 2.5 and 4.0 using HCl or NaOH before concentration. Due to the buffering capacity of the proteins, the initial pH value (pH before concentration) was set at 0.2 unit above the final desired value. In parallel, a wine sample was stored at 20 °C during 1 year.

Stability Experiments: Aggregation Kinetics by Dynamic Light Scattering (DLS). DLS experiments were carried out with a Malvern Autosizer 4700 (40 mW He−Ne laser, λ = 633 nm, APD detection, Malvern Instruments, Malvern, U.K.) and a Malvern Autosizer 3000 HS (6 mW He−Ne laser, λ = 633 nm, APD detection). Measurements were conducted at an angle of 90° from the incident beam. Sample evolution was followed by measurements of the scattering intensity and of the hydrodynamic diameter of particles. Each measurement represented the average of 10 subruns, and each sample was studied in duplicate. The autocorrelation function of the scattered light was analyzed using the cumulant method, which gives an average value of the aggregate hydrodynamic diameter (D_h) and the polydispersity index (PI) of the dispersion ($0 < PI < 1$). For studies at ambient temperature, DLS experiments were performed at 25 °C during the first 24 h to check the colloidal stability/instability and to follow aggregation kinetics for unstable samples. For studies at 40 and 70 °C, samples were first introduced in the measurement cell at a temperature of 25 °C, and control measurements were done before heating. The temperature was then raised to the heat treatment record value by means of the "Peltier block" associated with the Autosizer 3000 HS and maintained during 2 h. Following this heating step, the temperature was lowered to 25 °C. Aggregation was followed by DLS during heating and after cooling.

After DLS experiments, all samples (2 mL) were stored during 15 days at 20 °C before haze estimation (absorbance values at 720 nm and visual observation), separation of the precipitates by centrifugation (13000*g*, 15 min, 4 °C), and analysis of proteins. Nonprecipitated proteins were determined through SDS-PAGE analyses of the supernatants, performed as described before for the wine and the purified protein fraction. Centrifugal filter devices (3 kDa, Millipore) were used when needed to reach a final theoretical protein concentration in the sample of about 0.8 g L^{-1} and/or to remove salts before gel electrophoresis.

Thermal Stability of Protein and Reversibility by Differential Scanning Calorimetry (DSC). DSC experiments were performed with a VP-DSC instrument from GE Healthcare. The instrument was first equilibrated with tartaric acid buffer (2 g L^{-1}) at a given pH (between 3.0 and 4.0), adjusted with NaOH. The ionic strength was

set at 0.02 M with NaCl. Pool of purified wine proteins at a concentration of 0.16 g L^{-1} was analyzed using a temperature ramp from 20 to 100 °C at 1 °C min^{-1}. Proteins were dissolved in a tartaric acid buffer (2 g L^{-1}) at different pH values (between 3.0 and 4.0) and at an ionic strength of 0.02 M. Reversibility tests were performed by the repetition of the method. All solutions were degassed under vacuum before loading into DSC cells. Software Origin 7.0 was used for results analysis. Experiments were done twice.

RESULTS AND DISCUSSION

Wine Proteins. Protein concentration in the Sauvignon wine was estimated by weighing the protein fraction purified by ion exchange chromatography as being 0.16 g L^{-1}. The protein composition of the fraction was compared to that of the starting wine by 1D SDS-PAGE (Figure 1a). Proteins within each band were identified by mass spectrometry (Table 1). In accordance with literature data, these proteins were mainly thaumatin-like proteins (bands 5−9), chitinases (bands 3 and 4), β-glucanases (bands 2 and 3), and invertases (band 1). Bands 2 and 3 were lost during the purification steps. Thaumatin-like proteins represented the majority of the total protein content in the purified fraction (Figure 1a). In agreement with previous works,[3,11,16] most of the wine proteins were acidic, with isoelectric points between 4.5 and 5.0 (Figure 1b). Isoforms with higher isoelectric points (mainly between 5.0 and 6.0) were also found below 30 kDa.

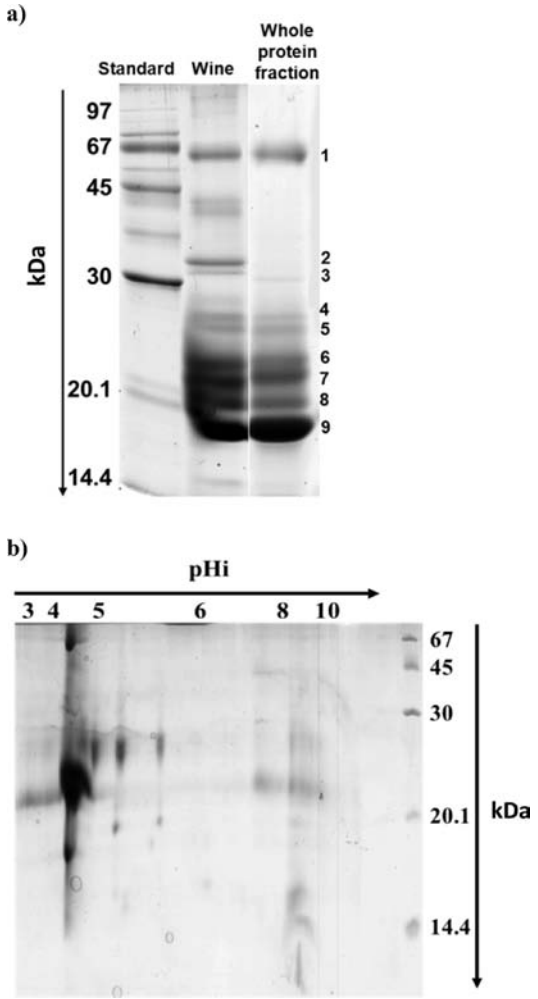

Figure 1. (a) 1D SDS-PAGE profile of the Sauvignon wine (molecular weight (MW) standards on left) and of the purified protein fraction. Protein amounts in the different bands of the initial wine and of the purified protein fraction were estimated by image analysis. In the initial wine the proportions were around 4.6% (band 1), 3.6% (band 2), 1.2% (band 3), 4.9% (band 4), 4.2% (band 5), 20.2% (band 6), 17.3% (band 7), 19.8% (band 8), and 24.2% (band 9). In the purified protein fraction the proportions were 9.0% (band 1), 3.0% (band 4), 5.0% (band 5), 12.0% (band 6), 17.5% (band 7), 15.5% (band 8), and 38.0% (band 9). (b) 2D SDS-PAGE profile of the purified fraction.

Wine Protein Stability at Room Temperature (25 °C). Colloidal instabilities, observed in white wines stored at appropriate temperatures, develop according to very slow kinetics,[5−7] likely in relation with the low concentrations of the involved components. Indeed, aggregation kinetics are not only dependent on the interactions between macromolecules but also strongly influenced by the time for these macromolecules to collide by diffusion.[29] To accelerate the kinetics, model protein solutions at concentrations higher than those encountered in wines were used first at ambient temperature to study the impact of the pH and ionic strength on their stability.

Model Systems. Concentrations in model solutions were 0.16 g L^{-1} (*C*), 0.8 g L^{-1} (*C**5), and 8 g L^{-1} (*C**50), *C* being the protein concentration in the initial wine. The pH was varied within the range 2.5−4.0, at two ionic strengths: 0.02 and 0.15 M. These ranges, larger than those usually encountered in white wines (pH range, 2.9−3.6; ionic strength range, 0.02−0.1 M), were selected to emphasize the effect of these two parameters. Increasing concentrations, by increasing kinetics, allowed first evidence of the strong impact of the pH and ionic strength on the stability of some of the wine proteins. This is illustrated in Figure 2 by DLS results obtained in model solutions at pH 3.0 and 4.0 and at 0.15 M. At pH 4.0, wine proteins were apparently stable: the scattered intensity remained constant and proportional to the protein concentration (Figure 2c). No aggregation was detected during the experiment length (24 h), even at 8 g L^{-1}, and no visible haze could be observed after 15 days. By contrast, at pH 3.0 (Figure 2a,b), aggregate formation was evident from a protein concentration of 0.16 g L^{-1}. The intensity scattering (I_s) remained low and constant during the first 2 h. This lag period was followed by a regular and progressive increase of I_s, indicating the onset of aggregation. Aggregation led within 12 h to the formation of micrometer-sized and highly polydisperse aggregates. It is worth noting that no haze was visible even after 15 days at room temperature, indicating that only a few aggregates had formed. Increasing the protein concentration to 0.8 and 8 g L^{-1} resulted in shorter lag periods and increased aggregation rates. At 0.8 g L^{-1}, micrometer-sized polydisperse aggregates formed within the first 8 h, leading to visible haze at the end of the DLS experiment. At 8 g L^{-1}, large aggregates, prone to sedimentation (decrease of I_s), formed within the first 4 h. DLS experiments were no longer suitable to follow aggregation kinetics.

Results obtained at all pH values for the two ionic strengths are detailed in Figure 3 for a concentration of 0.8 g L^{-1}. Results obtained at 0.16 and 8 g L^{-1}, which led to the same conclusions, are not shown. At 0.15 M, significant aggregation was observed only for pH ≤3.2. At pH 3.2, this aggregation remained moderate. It started only after a lag period of 1 h. After that, scattering intensity and aggregate average size evolved very slowly during the experiment. Aggregates remained submicronic. Lowering the pH to 3.0 and 2.5 strongly enhanced protein aggregation. The effect of the pH was evident for the two studied ionic strengths. However, the ionic strength strongly influenced the pH at which aggregation started and aggregation kinetics. Contrary to the observation at 0.15 M, aggregation could be evidenced by DLS at 0.02 M from pH 3.5.

Table 1. Protein Identification by Mass Spectrometry

spot	score[a] (peptide no.)	accession no. (Uniprot KB)	mass (Da) theoretical	mass (Da) measured[b]	name
1	158 (3)	Q9S944_VITVI	71786	70900	vacuolar invertase 1, GIN1
2	304 (6)	Q9M3U4_VITVI	37489	38600	β 1-3 glucanase
3	176 (3)	B5M495_VITVI	32181		class III chitinase
	110 (2)	B2ZP02_VITVI	37622	36000	β 1-3 glucanase
4	134 (1)	Q7XAU6_VITVI	28366	27500	class IV chitinase
	93 (1)	A5ASS2_VITVI	27884		putative uncharacterized protein
5	151 (2)	Q9M4G6_VITVI	24947	26000	putative thaumatin-like protein
6	151 (2)	Q9M4G6_VITVI	24947	23600	putative thaumatin-like protein
7	327 (6)	O04708_VITVI	24866		VVTL1
	274 (5)	Q9M4G6_VITVI	24947	22400	putative thaumatin-like protein
	208 (3)	P93621_VITVI	24828		osmotin-like protein
8	546 (9)	Q9M4G6_VITVI	24947		putative thaumatin-like protein
	297 (4)	A5AHJ5_VITVI	25082	20400	putative uncharacterized protein
	158 (2)	A3QRB4_VITVI	24817		thaumatin-like protein
9	451 (7)	O04708_VITVI	24866	18500	VVTL1
	178 (3)	A3QRB5_VITVI	24888		thaumatin-like protein

[a]Identification score along with the number of peptides (in parentheses) that matched with peptides of the Uniprot database. [b]Obtained from 1D-SDS PAGE electrophoresis.

In addition, lowering the pH induced an immediate increase in scattering intensity. This was related to the immediate formation of "polydisperse" colloidal particles with mean hydrodynamic diameters D_h between 200 and 300 nm. After that, I_s did not evolve strongly, whereas aggregate size kept increasing regularly. This indicates a very quick aggregation, followed by interactions between the aggregates and particle growth. Comparison between final visual hazes is given Table 2.

Proteins involved in aggregation were identified for all of the tested conditions after 15 days at 20 °C. Results obtained at a protein concentration of 0.8 g L^{-1} are shown Figure 4a (see Supporting Information Figure S1 for protein quantification). Precipitated proteins were found within the range of 22−28 kDa (bands 4−7) and were previously identified as thaumatin-like proteins and chitinases. Although no aggregation could be detected by DLS during the first 24 h or by observation after 15 days, SDS-PAGE analyses indicated that some proteins in bands 4 and 5 had precipitated at pH 4. This is likely related to the low concentration of these proteins, leading to very slow aggregation kinetics and haze undetectable to the eye. The 22−24 kDa proteins were significantly affected at low pH (<3.2). Their precipitation was enhanced at the highest ionic strength. Another important result was that most of the wine proteins remained stable within the pH and ionic strength range tested: the 19 and 20 kDa thaumatin-like proteins and the invertases (71 kDa), which represent about 63% of the whole initial protein content in the fraction (Figure 1a), were affected neither by the lowest pH nor by the ionic strength.

Protein aggregation in solution is dependent on both conformational and colloidal stability. It can occur due to attractive intermolecular colloidal interactions and involve native states. It can also be a two-step phenomenon, including first a loss of the initial conformation, which can be more or less reversible, followed by irreversible aggregation of the unfolded/partly unfolded species.[25] Considering their isoelectric points, wine proteins are all positively charged within the tested pH range, and their overall positive charge either remains constant or increases when the pH decreases from 4.0 to 2.5. Intermolecular electrostatic repulsions are thus expected to contribute to their colloidal stability. The increasing instability observed for the 22−28 kDa proteins when the pH was lowered was thus likely related to a two-step mechanism, involving conformational changes (pH unfolding) and formation of aggregation-prone intermediates, followed by colloidal aggregation. Indeed, pH determines protein charge and thus intramolecular electrostatic interactions between charged groups. At acidic pH, increasing repulsive interactions between charged groups destabilize the folded conformation, which in turn provokes changes in the surface area and in the zones accessible for intermolecular interaction.[25,30] Decreasing the pH, by increasing the degree of unfolding, may be responsible for the increased aggregation and aggregation rates observed in the present results.

The impact of the ionic strength is more complex. Considering a two-step mechanism, that is, structural changes followed by colloidal interactions, aggregation kinetics for a given protein concentration will be controlled by the unfolding rate and the colloidal aggregation rate. It appeared from our results that these two steps are differently affected by the ionic strength. The different behaviors observed in the initial aggregation at different ionic strengths can be related to differences in the degree of unfolding. Indeed, electrostatic repulsions between charged segments of a protein have been shown to affect the degree of unfolding, leading to more or less extended conformations and different aggregation rates.[30] Degree of unfolding due to electrostatic effects is expected to be screened when the ionic strength increases, leading to less extended conformations and thus smaller accessible surface areas.

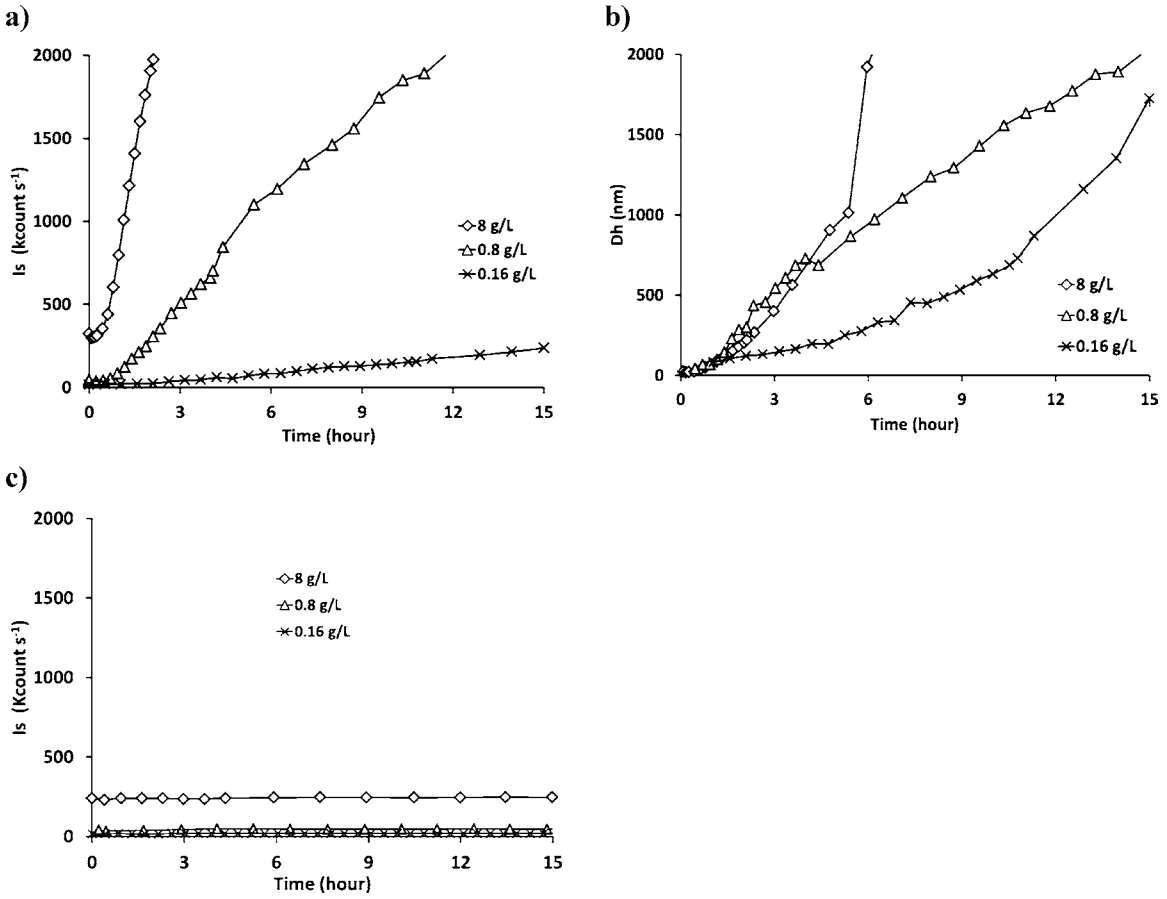

Figure 2. DLS experiments performed on Sauvignon wine proteins in model wine-like solutions at 25 °C and for different protein concentrations: 8 g L^{-1} (◇), 0.8 g L^{-1} (△), 0.16 g L^{-1} (×). Experiments were followed during 24 h and repeated twice. For the sake of clarity, only one kinetic and the first 15 h are shown for a given concentration. (a) Light scattering intensity I_s (kcounts s^{-1}) and (b) average hydrodynamic diameter (D_h, nm) at 0.15 M and pH 3.0. Standard deviations between two experiments were less than 10 and 15% for scattered light intensity and average hydrodynamic diameters, respectively. (c) Light scattering intensity I_s (kcounts s^{-1}) at 0.15 M and pH 4.0.

Apart from this effect on the onset of aggregation, the ionic strength impact on colloidal intermolecular interactions was in accordance with the prevision of the extended DLVO theory:[31] as the ionic strength increased, the net electrostatic repulsive contribution to colloidal protein−protein interactions decreased, enhancing aggregation (Figures 3 and 4a; Table 2).

Wine. Considering the complexity of wine composition, non-protein compounds may interfere with protein stability and be involved in haze development. Thus, the presence of polysaccharides and polyphenols in "natural" haze has been shown.[5] Different experiments were performed to compare results obtained in model solutions with protein behavior in the initial wine (pH 3.2, ionic strength on the order of 0.02 M and protein concentration of 0.16 g L^{-1}). In a first set of experiments, the wine was concentrated at 20 °C using osmotic stress and a membrane cutoff of 3.5 kDa. When the results with the experiments in model solutions are compared, it is important to note that when using this technique not only the wine proteins but also all of the components with molecular weight (MW) >3.5 kDa (especially polysaccharides) were concentrated. A concentration factor of 5 was applied, the wine pH being adjusted between 2.5 and 4.0. As in model solutions, decreasing the pH enhanced protein precipitation. SDS-PAGE analysis (Figure 4b and Supporting Information Figure S1) indicated that the same protein bands as in model solutions (4−7) were involved in aggregation. Other information, by comparison with model solutions, was the impact of the pH on bands 2 and 3 (36 and 38.6 kDa, class III chitinase and β-glucanases). These proteins, lost during the purification steps (Figure 1a), were precipitated from pH 3.5 (band 3, 36 kDa) and 3.0 (band 2, 38.6 kDa). However, some differences were noted when experiments were performed in wine: bands 4 and 5 at 26−28 kDa were fully precipitated at all pH values, and, contrary to that observed in model solutions, a visible haze was observed whatever the pH (Table 3). The same experiments repeated on a protein-free wine showed that proteins were needed to induce hazing. These differences in hazing are likely related to the involvement of other compounds in the wine, modulating aggregation. After 1 year of storage at 20 °C, a "natural" haze had formed in the Sauvignon wine. Once again, bands 3−7 (β-glucanases, chitinases, and some thaumatin-like proteins) were precipitated, whereas 19 and 20 kDa thaumatins (band 8 and 9) and invertases (71 kDa, band 1) remained unaffected (Figure 4c).

Combined Effect of Heat, pH, and Ionic Strength on Wine Protein Stability. The thermal stability of wine proteins was evaluated first at different pH values, using DSC (Figure 5a). Three major groups corresponding to different melting temperatures were observed at 56, 67, and 81 °C at pH 3.0. Recent studies[10] allow us to suppose that the first peak represented chitinases and some thaumatin-like proteins (melting temperature (T_m) at 55 and 56 °C, respectively), the second one thaumatin-like proteins (T_m = 62 °C), and the third one invertases (T_m = 81 °C). Increasing the pH within the range 3.0−4.0 enhanced

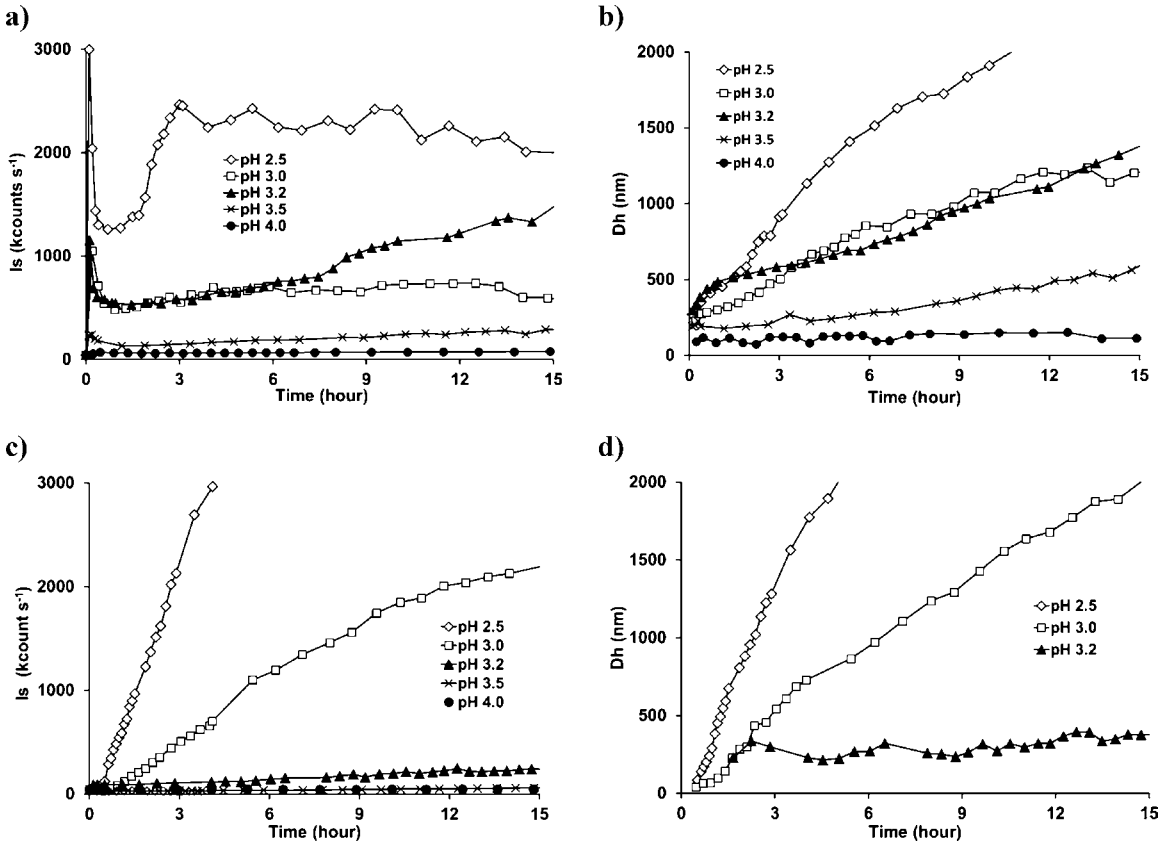

Figure 3. DLS experiments performed on Sauvignon wine proteins (0.8 g L^{-1}) showing the impact of the pH and ionic strength on their stability at 25 °C: (a) I_s (kcounts s^{-1}) and (b) D_h (nm) at 0.02 M. (c) I_s (kcounts s^{-1}) and (d) D_h (nm) at 0.15 M. Experiments were performed at two ionic strengths (0.02 and 0.15 M) and five different pH values: 2.5 (◇), 3.0 (□), 3.2 (▲), 3.5 (×), 4.0 (●).

Table 2. Sample Turbidity (Absorbance at 720 nm) and Visual Haze after 15 Days of Storage at Room Temperature of Model Systems with a Protein Concentration of 0.8 g L^{-1} (25 °C) and 0.16 g L^{-1} (2 h at 40 and 70 °C) Highlighting the Impact of pH and Ionic Strength[a]

		pH				
protein concn and temp	ionic strength (M)	2.5	3.0	3.2	3.5	4.0
0.8 g L^{-1}	0.02	0.031 ± 0.002 (++)	0.029 ± 0.001 (+)	0.020 ± 0.002 (+)	0.006 ± 0.002 (−)	0.003 ± 0.000 (−)
25 °C	0.15	0.037 ± 0.005 (++)	0.027 ± 0.001 (+)	0.002 ± 0.000 (−)	0.002 ± 0.000 (−)	0.002 ± 0.001 (−)
0.16 g L^{-1}	0.02	0.000 ± 0.000 (−)		0.007 ± 0.000 (+)		0.000 ± 0.000 (−)
2 h at 40 °C	0.15	0.026 ± 0.002 (+)		0.027 ± 0.001 (+)		0.003 ± 0.000 (−)
0.16 g L^{-1}	0.02	0.000 ± 0.000 (−)		0.004 ± 0.000 (−)		0.036 ± 0.000 (++)
2 h at 70 °C	0.15	0.022 ± 0.000 (+)		0.052 ± 0.000 (++)		0.072 ± 0.001 (+++)

[a](−) no haze visible to the eye; (+), (++), (+++) increasing haze visible to the eye.

protein thermal stability (Figure 5b): melting temperatures increased with the pH. It is worth noting that chitinases and thaumatin-like proteins (peaks 1 and 2) were more affected by the pH variation (ΔT_m = 8 °C) than invertases (peak 3) (ΔT_m = 3 °C). Further information is the impact of pH on the reversibility of protein thermal unfolding (Figure 5c). At pH 3.0, as shown by Falconer et al.,[10] thaumatin-like proteins (peak 2) showed some refolding until the fifth temperature ramp. However, in present work, no reversibility was observed at pH 4.0. Because of the protein isoelectric point (mostly around 4.0−5.0), proteins are less charged at pH 4.0 than at pH 3.0. It can be supposed that at low pH, electrostatic repulsions slow the aggregation of unfolded proteins. Some will be able to refold. At pH 4.0, electrostatic repulsions are lowered, leading to the quick aggregation of all proteins.

DLS experiments were performed at 40 and 70 °C to assess the combined effects of temperature, pH, and ionic strength on the colloidal stability. These two temperatures were chosen for different reasons. First, they correspond to temperatures that can sometimes be reached during transport or storage (40 °C) or commonly used in heat tests (70 °C). Second, in a previous work, different aggregation mechanisms had been highlighted for these two temperatures.[9] DLS experiments were done both on wine and on model systems using the same protein concentration as in the initial Sauvignon wine. Considering the previous results, three pH values (2.5, 3.2 and 4.0) were selected for the model solutions (ionic strengths of 0.02 and 0.15 M). Heat treatment duration was set to 2 h and aggregation followed by DLS during heating and cooling.

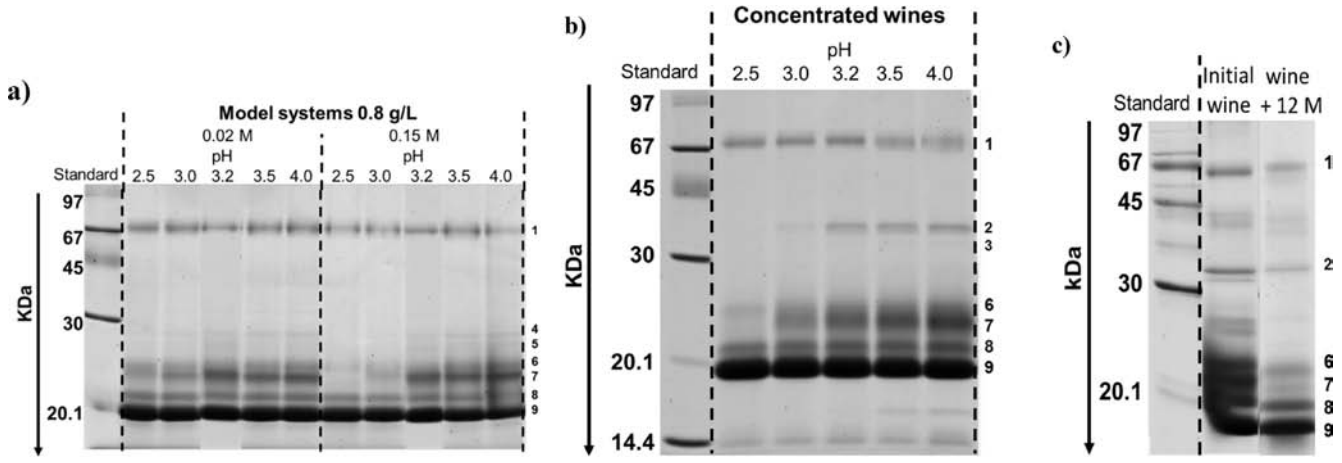

Figure 4. 1D SDS-PAGE profiles from left to right: (a) MW standards, supernatant of protein model solutions (0.8 g L^{-1}), at different pH values and ionic strengths, after DLS experiments at 25 °C (24 h) and 15 days of storage at 20 °C; (b) MW standards, wine concentrated in components with MW higher than 3.5 (proteins and polysaccharides, concentration factor 5) after 15 days of storage at different pH values and at 20 °C; (c) MW standards, initial wine, and wine after 12 months of storage at 20 °C.

Table 3. Sample Turbidity (Absorbance at 720 nm) and Visual Haze after 15 Days of Storage at Room Temperature Observed for Concentrated Wine (25 °C) and Heat-Treated Wine (2 h at 40 and 70 °C) Highlighting the Impact of the pH[a]

	pH				
sample and temp	2.5	3.0	3.2	3.5	4.0
concentrated wine (×5), 25 °C	0.056 ± 0.003 (++)	0.032 ± 0.012 (++)	0.036 ± 0.005 (++)	0.035 ± 0.017 (++)	0.027 ± 0.011 (+)
wine, 2 h at 40 °C	0.004 ± 0.001 (−)	0.006 ± 0.001 (+)	0.009 ± 0.001 (+)	0.012 ± 0.003 (+)	0.003 ± 0.001 (−)
wine, 2 h at 70 °C	0.002 ± 0.002 (−)	0.020 ± 0.002 (+)	0.028 ± 0.002 (+)	0.049 ± 0.008 (++)	0.094 ± 0.015 (+++)

[a](−) no haze visible to the eye; (+), (++), (+++) increasing haze visible to the eye.

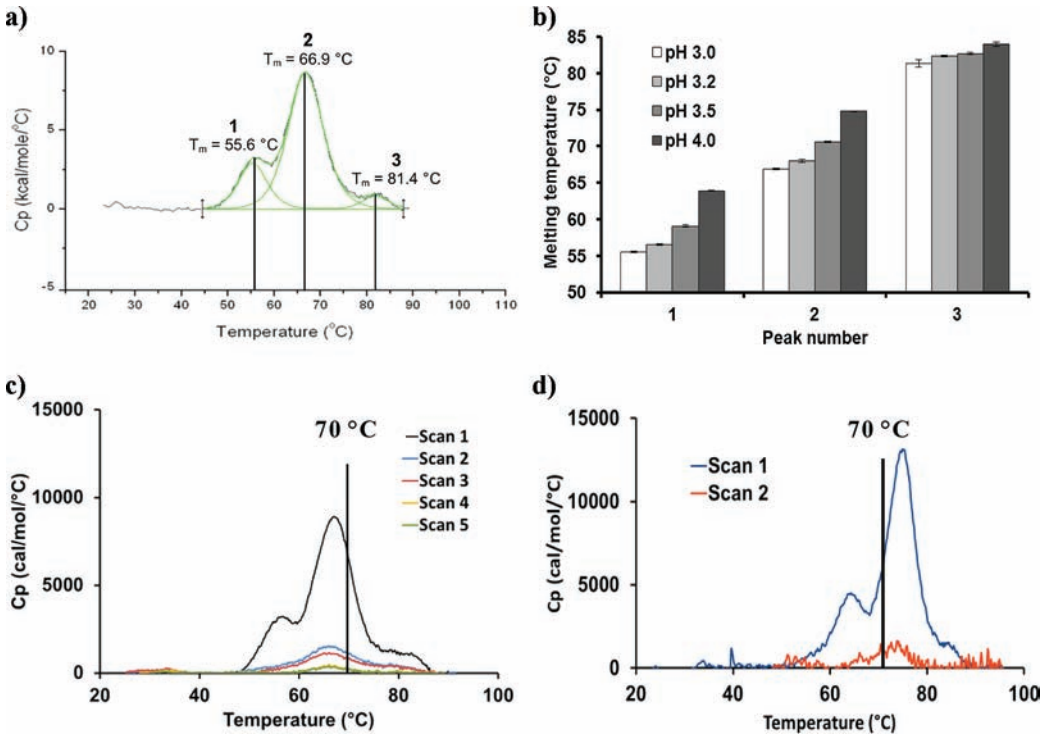

Figure 5. Determination of wine protein melting temperature by DSC. Experiments were performed on the purified protein fraction at different pH values (ionic strength, 0.02 M): (a) pH 3.0; (b) pH impact on protein thermal stability. Repeated DSC scans were performed on wine protein fraction at (c) pH 3.0 and (d) pH 4.0. Cp, thermal capacity at constant pressure.

Results obtained at 40 °C are shown Figure 6. In the wine (Figure 6a,b) and at pH 2.5, only a very small increase of the scattering intensity was observed during the heating step. Aggregation mainly developed during cooling. I_s quickly stabilized at a plateau value, whereas aggregate size kept increasing progressively to a mean D_h of 500 nm. Because

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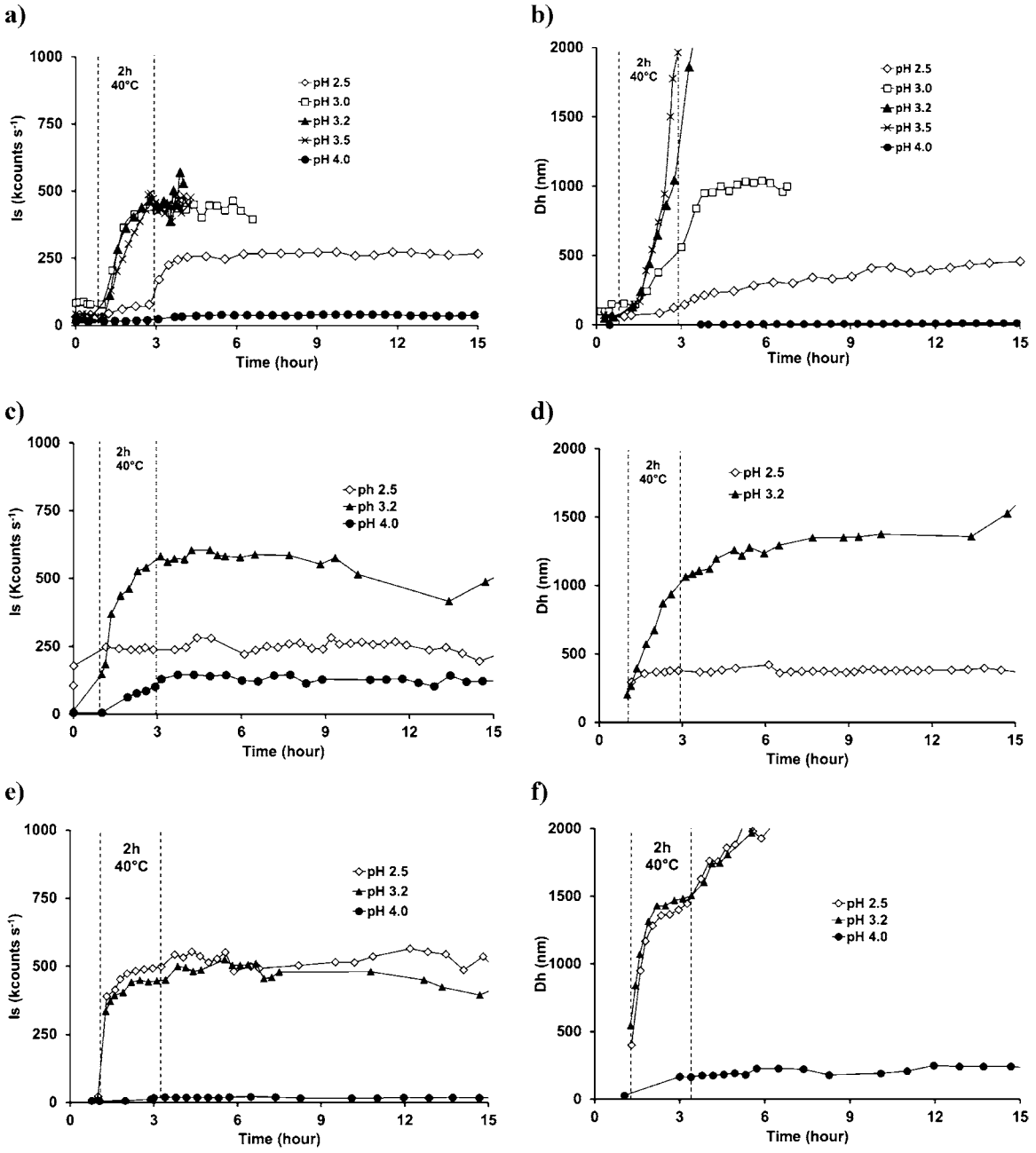

Figure 6. Aggregation kinetics in the Sauvignon wine and model protein solutions (0.16 g L^{-1}) followed by DLS during a 2 h heat treatment at 40 °C and cooling at 25 °C (the temperature of 25 °C was reached within 10 min). Different pH values were studied: 2.5 (◇), 3.0 (□), 3.2 (▲), 3.5 (×), 4.0 (●). Results were obtained on wine ((a) I_s (kcounts s^{-1}) and (b) D_h (nm)), model solutions at 0.02 M ((c) I_s (kcounts s^{-1}) and (d) D_h (nm)), and model solutions at 0.15 M ((e) I_s (kcounts s^{-1}) and (f) D_h (nm)).

of the small size of the aggregates, no haze was visible after 15 days at 20 °C (Table 3). When the pH was increased to 3.0, 3.2 and 3.5, which correspond to pH values usually encountered in wines, aggregation occurred as soon as the temperature was set to 40 °C. Greater aggregation, leading to the formation of micrometer-sized aggregates, occurred during the heating step and was quickly followed by precipitation and the formation of a visible haze. For a close value of I_s, aggregate size increased more quickly when the pH increased from 3.0 to 3.5. Proteins involved in aggregation between pH 2.5 and 3.5 were the same as those precipitated at room temperature at pH ≤3.2, that is, β-glucanases, chitinases, and the 22−24 kDa thaumatin-like proteins (Figures 4 and 8a). At pH 4.0, the heating/cooling cycle affected the scattering intensity but to a very low extent, and only very small aggregates formed during cooling (D_h from 40 to 100 nm). After 15 days, no haze was visible and SDS-PAGE analysis did not show detectable protein depletion, indicating that protein aggregation was very limited at this pH (Figure 8a). DSC results also indicate a combined effect of the pH and temperature on the protein conformational stability: a significant increase of the T_m was observed between pH 3.0 and 4.0. This increase was of 8 °C for peaks 1 and 2 and of 3 °C for peak 3. This means that at pH 4.0, proteins will be less affected by thermal unfolding than at pH 3.0, in accordance with DLS and SDS-PAGE results. According to DSC experiments, unfolding starts at pH 3.0 when the temperature reaches 48 °C. However, in DSC, the temperature is increased according to a "ramp": it takes 20 min to increase the temperature from 20 to

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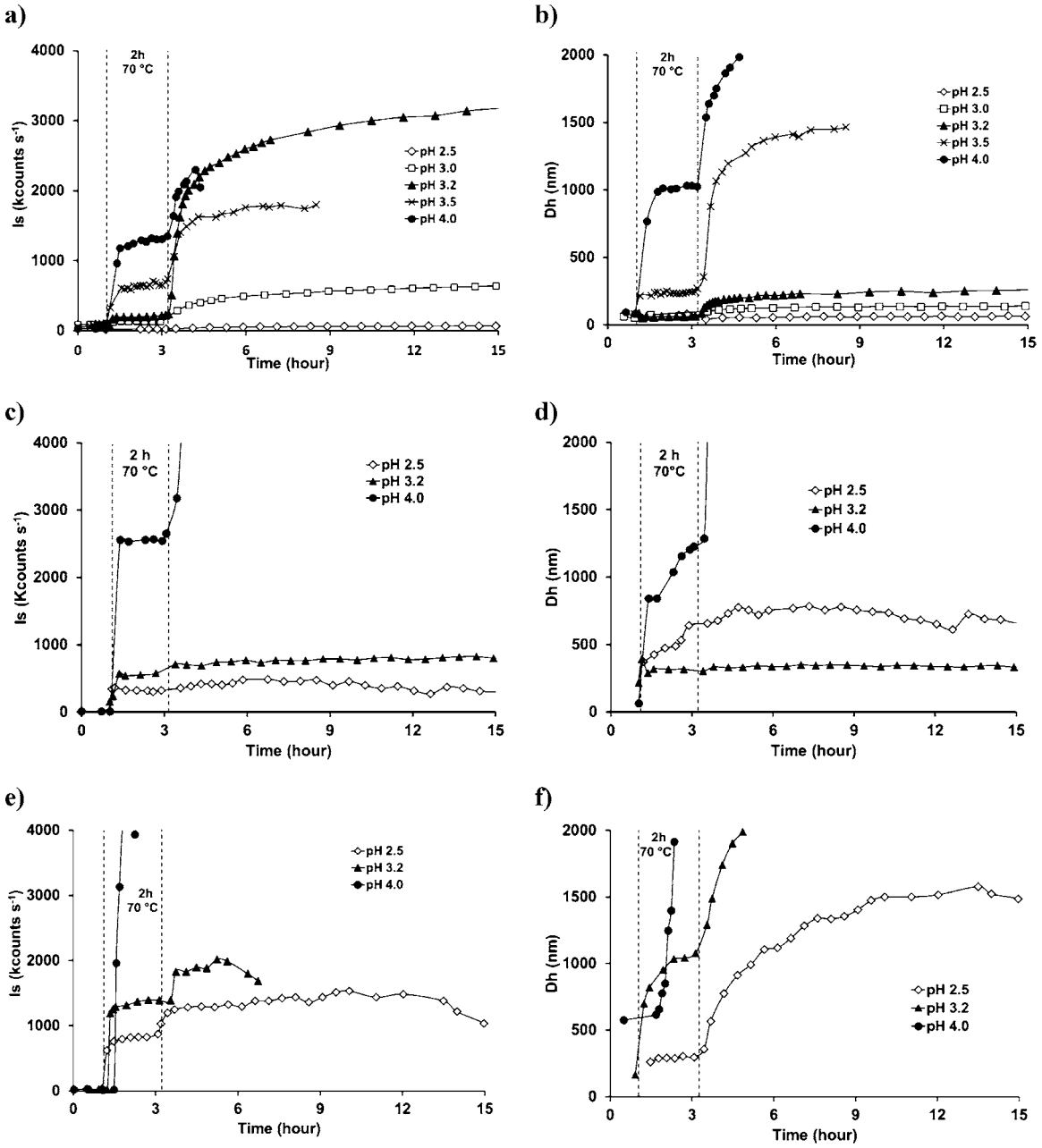

Figure 7. Aggregation kinetics in the Sauvignon wine and model protein solutions (0.16 g L^{-1}) followed by DLS during a 2 h heat treatment at 70 °C and cooling at 25 °C (the temperature of 25 °C was reached within 15 min). Different pH values were studied: 2.5 (◇), 3.0 (□), 3.2 (▲), 3.5 (×), 4.0 (●). Results were obtained on wine ((a) I_s (kcounts s^{-1}) and (b) D_h (nm)), model solutions at 0.02 M ((c) I_s (kcounts s^{-1}) and (d) D_h (nm)), and model solutions at 0.15 M ((e) I_s (kcounts s^{-1}) and (f) D_h (nm)).

40 °C. In the case of heat treatment, a temperature of 40 °C is applied during 2 h. As unfolding is a kinetic phenomenon, the most thermally unstable proteins probably start unfolding below 48 °C.[10,25]

The different aggregation kinetics, final aggregate characteristics, and final haze as a function of pH evidenced by DLS between pH 2.5 and 3.5 are thus attributable to colloidal interactions. Changes in the overall charge of the proteins between pH 2.5 and 3.5 likely strongly modify electrostatic interactions between unfolded species and/or aggregates and thus aggregation kinetics.[31,32] At pH 2.5, proteins are highly charged and so are the aggregates. Strong electrostatic repulsions between the latter, which increase with their diameter, prevent particle growth. These electrostatic repulsions decrease with the protein charge when the pH is increased. Results obtained in model solutions at two different ionic strengths (Figure 6c–f) confirm this hypothesis. At 0.02 M, proteins in model solutions behave as in wine: heat treatment at pH 2.5 led to the formation of metastable aggregates with a finite submicronic size, whereas enlarged aggregation occurred at pH 3.2. At 0.15 M, electrostatic repulsions were screened and no significant differences were observed between the two pH values. Precipitated proteins were the same as in wine (Figure 8b). To further validate this assumption, 0.1 M NaCl was added in the wine at pH 2.5, after the heat treatment and 2 h of cooling (Supporting Information Figure S2). This addition induced the precipitation of the metastable aggregates, thus confirming the role played by electrostatic repulsions in colloidal stability. This metastability (strong electrostatic repulsion between aggregates) at low pH was not

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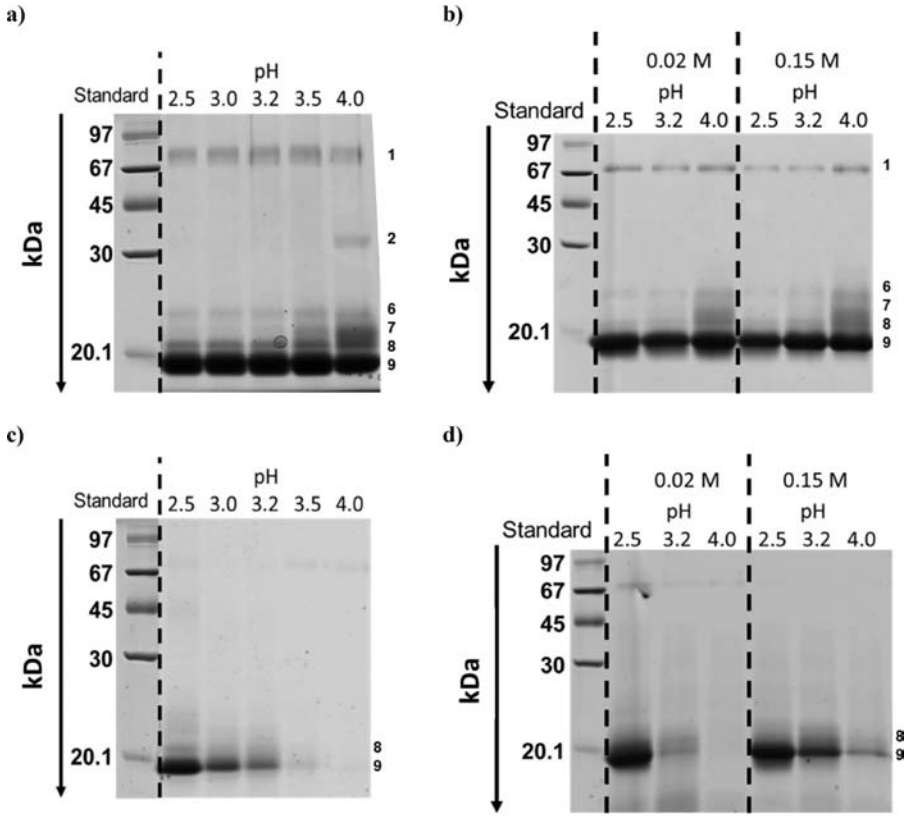

Figure 8. 1D SDS-PAGE profile, from left to right: (a) MW standards, wine after 2 h of heat treatment at 40 °C at different pH values; (b) MW standards, model solutions of wine proteins (0.16 g/L) after 2 h of heat treatment at 40 °C at different pH values and ionic strengths; (c) MW standards, wine after 2 h of heat treatment at 70 °C at different pH values; (d) MW standards, model solutions of wine proteins (0.16 g/L) after 2 h of heat treatment at 70 °C at different pH values and ionic strengths. Following DLS experiments, samples were stored for 15 days at 20 °C before removal of precipitated proteins and analyses of the supernatants.

observed at 25 °C, indicating a different charge of the aggregates formed at room temperature. It can be supposed that a combined effect of temperature and pH induces at 40 °C a higher degree of unfolding, leading to higher charge exposure of the aggregate-prone species and higher aggregate surface charges. Experiments on pure proteins are needed to confirm this hypothesis.

DLS experiments performed at 70 °C showed again the strong impact of the pH on aggregation kinetics, on the structure and size of the aggregates, and, thus, on final haze (Figures 7 and 8c; Table 3). However, and by contrast to that observed before, aggregation was regularly enhanced when the pH was increased and the fastest kinetics were observed at pH 4.0. These results are in accordance with the literature, where the incidence of the pH on protein hazing was studied using high-temperature treatments.[1,33] At such temperatures, DSC indicated that most of the wine proteins are strongly affected by heat whatever the pH. A 2 h heat treatment is expected to induce their complete unfolding. Aggregation will thus be driven by colloidal interactions and more particularly, as evidenced by our results, by electrostatic interactions. In wine and in model solutions at 0.02 M, aggregation and aggregate growth were prevented by electrostatic repulsion up to pH 3.2. At such pH, electrostatic repulsions prevented a class of thaumatin-like proteins (19−20 kDa) from being fully involved in aggregation and thus allowed them to partially refold, as shown by SDS-PAGE results (Figure 8c,d) and DSC (Figure 5c). When the pH was further increased, a decrease of the charge led to faster kinetics and induced the precipitation of all proteins. By comparison to that observed at 40 °C, a higher salt concentration (0.3 M) was needed to destabilize the aggregates formed at pH 2.5 (Supporting Information Figure S2). This indicates higher electrostatic repulsions between the aggregates formed at high temperature, likely related to higher surface charges. These differences in the charge properties of the aggregates formed at different temperatures may have two different origins: involvement in the aggregate structure of other proteins with higher charge and/or higher charge exposure due to a higher degree of unfolding of the proteins.

Figure 9 summarizes the whole results obtained and the mechanisms proposed to explain the impact of pH, ionic strength, and temperature on the conformational and colloidal stabilities of wine proteins. The present results indicated that wine stability at low and intermediate temperatures (temperatures compatible with nonrefrigerated transport or storage) will be strongly influenced by its content in proteins with low conformational stability, rather than by its total protein concentration. These proteins were found to be chitinases but also β-glucanases and some thaumatin-like isoforms. In addition, results also demonstrated the strong part played by the pH on the conformational stability of these proteins: they strongly suggest pH-induced unfolding, leading to conformational changes responsible for their colloidal aggregation at low pH. This hypothesis of pH-induced unfolding will be studied in detail using pure proteins. Within the wine range (2.9−3.6) and for a given protein composition, pH will thus influence

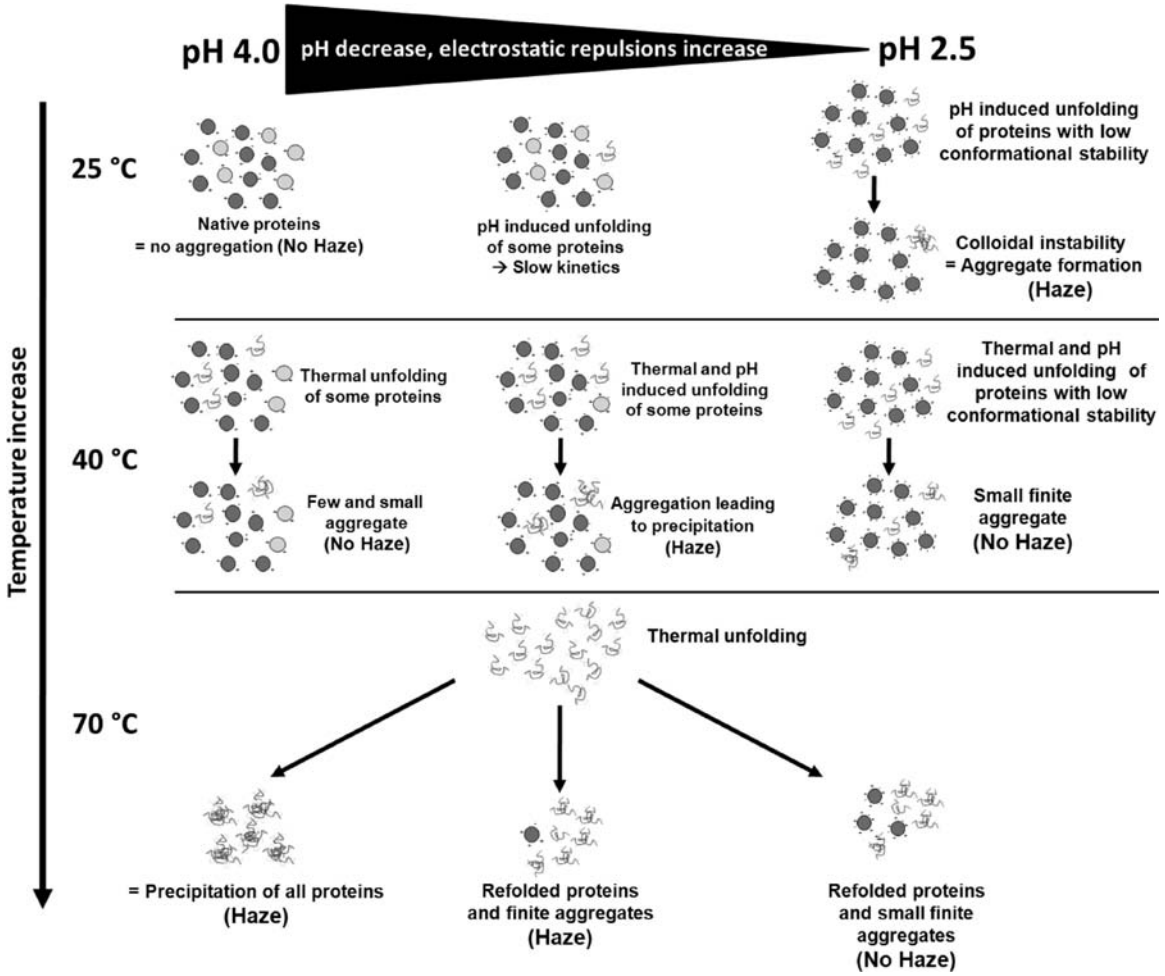

Figure 9. Proposed mechanisms for protein aggregation depending on the pH and the heat treatment, illustrating the combined effect of pH and temperature on protein aggregation. At ambient temperature, some proteins with low conformational stability (chitinases, β-glucanases, and 22–24 kDa TLP isoforms) precipitate at low pH ($\leq$3.2) due to pH-induced unfolding. The latter is attributable to enhanced intramolecular electrostatic repulsions when the overall protein charge increases. When the temperature reaches 40 °C, a combined effect of the pH and temperature accelerate the unfolding rate of the same proteins. Aggregation was observed for pH $\leq$3.5 and led to different final hazes due to electrostatic repulsions between aggregates (maximum at low pH). At 70 °C, most proteins are heat-unfolded. Aggregation and final haze are governed by electrostatic interactions between proteins (intermolecular) and aggregates, which strongly decrease when the pH increases.

the long-term stability of wines with regard to protein haze. The determinant part played by electrostatic interactions in the colloidal stability of wine proteins and thus on final haze was also evidenced. Nonprotein compounds in wines such as polysaccharides, polyphenols, and specific anions are considered as having a strong influence on protein haze. However, the present results indicate that wine pH and ionic strength likely significantly contribute to this variability. Totally different results in terms of aggregation and haze formation were obtained when different temperatures were tested. Considering different mechanisms (pH and heat-induced unfolding) and the importance of charges on conformational and colloidal stability allowed us to explain these apparently conflicting results. This point emphasizes the importance of considering different temperatures for the identification of mechanisms involved in protein hazing.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

DLS, dynamic light scattering; DSC, differential scanning calorimetry; I_s, scattering intensity; D_h, hydrodynamic diameter; T_m, melting temperature; C_p, thermal capacity at constant pressure.

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