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Protein Aggregation in White Wines: Influence of the Temperature on Aggregation Kinetics and Mechanisms

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High temperatures (typically 80 °C) are widely used to assess wine stability with regard to protein haze or to study mechanisms involved in their formation. Dynamic light scattering experiments were performed to follow aggregation kinetics and aggregate characteristics in white wines at different temperatures (30−70 °C). Aggregation was followed during heating and cooling to 25 °C. Results were coupled with the study of the time−temperature dependence of heat-induced protein aggregation. At low temperature (40 °C), aggregation developed during heating. Colloidal equilibria were such that attractive interactions between species led to the rapid formation of micrometer-sized aggregates. At higher temperatures (60 and 70 °C), enhanced protein precipitation was expected and observed. However, high temperatures prevented aggregation, which mainly developed during cooling. Depending on the wine, cooling induced the formation of sub-micronic metastable aggregates stabilized by electrostatic repulsions, or the rapid formation of micrometer-sized aggregates, prone to sedimentation.

KEYWORDS: wine proteins; temperature; aggregation kinetics; colloidal stability

INTRODUCTION

Knowledge of physicochemical mechanisms involved in protein aggregation and precipitation is of great importance in enology to control the stability of white and rosé wines. Though present in rather small amounts, typically from 15 to 300 mg/L, proteins may be responsible for the development of hazes or deposits in bottled wines (*1*−*3*). This common defect makes wine unacceptable for the consumer. Protein stabilization is currently achieved by means of their adsorption on bentonite (bentonite fining). Wine stability is commonly assessed using heat-precipitation tests and the bentonite dose is adjusted as a function of the response to these tests (*3*−*5*). Though effective, bentonite fining causes substantial wine losses (*6*) and may adversely affect wine quality due to significant aroma adsorption (*7*). In addition, both bentonite handling and disposal of spent bentonites raise safety and environmental issues. In this context, there is an interest for the development of more reliable predictive tools and/or alternative stabilization technologies, which maintain wine quality while reducing costs and environmental impact (*8*−*10*). This interest has resulted in increasing research work devoted to this problem.

Special attention has been paid to the identification of white wine proteins. Major proteins have been identified as being pathogenesis-related (PR) proteins including thaumatin-like proteins, osmotins, and chitinases (*11*−*13*). These proteins have molecular weights (M_w) ranging from 20 to 30−35 kDa and are usually acidics. Also often present in wines are grape vacuolar invertases ($M_w \approx 66$ kDa) and as yet unidentified low MW proteins (< 15 kDa). The presence of grape β-glucanases, with a molecular weight within the range 37−41 kDa, has also been reported (*14*, *15*). By comparing simple nonreducing one-dimensional (1D) electrophoresis profiles of different white wines, it could be concluded that their protein fraction is essentially composed of a small number of polypeptides and that they mainly differ between each other in their respective amounts of these different protein classes. However, a more detailed analysis and the use of different separation methods show that this simplicity in the protein composition is only apparent (*12*, *16*, *17*). The existence of a huge diversity of structurally similar but distinct polypeptides in wines, due to the existence of microheterogeneities between these polypeptides, has been demonstrated (*12*). This diversity was first attributed to a limited proteolysis of a common precursor during grape ripening or winemaking. Later studies indicated that PR-proteins are synthetized in grape berries in a wide variety of forms from the earlier stages of the maturation (*11*).

Protein instability in wines is attributed to their slow unfolding under unsuitable storage conditions (especially excessive temperatures), leading to their aggregation and precipitation (*1*, *4*, *18*). It thus involves two distinct phenomena: protein unfolding, due to changes in intramolecular interactions, and colloidal aggregation, related to intermolecular interactions. The structural diversity of wine proteins, that can lead to different conformational and colloidal stabilities, is in itself an important point to account for in the identification of the physicochemical mechanisms

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Table 1. Conventional Enological Analyses of the Sauvignon and Chardonnay Wines

	Sa1	Sa2	Ch
ethanol (%v/v)	11.5	11.5	13.7
pH	3.17	2.99	3.41
titratable acidity (g H_2SO_4/L)	4.9	4.4	3.6
total SO_2 (mg/L)	87		
free SO_2 (mg/L)	25	10	13
total polyphenol index	4.6	7.3	7.2
K^+ (mg/L)	566	533	788
Na^+ (mg/L)	12	5	8
Ca^{2+} (mg/L)	80	43	40
Mg^{2+} (mg/L)	70		

involved in haze development. In addition, factors in wines other than protein composition are likely to modulate their sensitivity to protein haze. Haze formation has been shown to be strongly affected by the presence of nonprotein compounds such as polyphenols (*19*, *20*), ions (*21*, *22*), and polysaccharides (*23*−*25*). Protein stability/instability in wines is thus a complex problem. Issues of (i) the influence of wine composition (pH, ethanol, ionic strength and ionic content, cosolutes), (ii) the role of the conformational versus colloidal stability as a function of this composition, and (iii) the influence of the diversity of the protein isoforms, are not solved yet. Most studies have been performed using high heating temperatures (typically between 60 and 80 °C), which allows acceleration of protein unfolding rates. However, protein heat-induced aggregation may involve different physicochemical mechanisms when different temperatures are considered (*26*). This is likely the case when dealing with a complex medium such as wine that contains different protein classes with different thermal stabilities (*1*, *15*, *27*). It is thus of importance to consider different situations, as storage temperatures are hardly likely to reach values as high as the ones used in common stability tests. Besides, only the final aggregation is taken into account in most cases, thanks to turbidity measurements performed following heat exposure and at least one night at room temperature. This does not provide information concerning aggregation kinetics and possibly makes the comparison between different wines more difficult. The aim of the present work was to get some information concerning the relationships between the temperature experienced by a wine and the ensuing aggregation kinetics and mechanisms. To this end, we investigated, at different heat treatment temperatures and utilizing dynamic light scattering experiments (DLS), the earlier protein aggregation kinetics in different wines (i.e., aggregation kinetics during the heating and initial cooling steps). 1D SDS−PAGE coupled with image analysis was used to estimate the protein contents of the different wines and their depletion due to heat-induced aggregation. Depletion was followed for different time−temperature heat treatments and compared to DLS results.

MATERIALS AND METHODS

Wines. The white wines used in the present study were elaborated in 2007 and 2009 from Sauvignon and Chardonnay varieties at the Pech-Rouge Experimental Unit (INRA, Gruissan, France). No bentonite fining was performed. Conventional enological parameters were analyzed according to the Vine and Wine International Organisation methods (**Table 1**).

Protein Quantification Using 1D Electrophoresis Coupled with Image Analysis. Protein analyses were performed using 1D SDS−PAGE coupled with image analysis, according to the procedure described before (*15*). In this procedure, proteins are first isolated and concentrated 10-fold thanks to a first adsorption/desorption step using bentonite. To this end, bentonite was added to a 1 mL wine aliquot (final bentonite concentration: 200 g/Hl). After 30 min under soft shaking the mixture was centrifuged for 15 min at 20000*g* and at 4 °C. The supernatant was removed and 100 μL of Laemmli buffer was added to the pellet to allow protein desorption. The suspension was shaken during 15 min and the bentonite was separated by centrifugation, as described before. Proteins in the buffer were then concentrated 10-fold by comparison to the original wine. They were separated by 1D SDS−PAGE, performed in 14% acrylamide resolving gel (resolving 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 then stained with Coomassie blue R-250 (Biorad) and scanned at 300 dpi with an Image scanner (GE Biosciences). Image elaboration and analysis were carried out with the Totallab software (Nonlinear Dynamics Ltd.). Image analysis was used to calculate the "volume" of each staining band from sample. The volume of the bovine serum albumin band of the low molecular weight calibration kit was used as a standard and results are expressed in mg equivalent BSA/L.

Time−Temperature Dependence of Protein Heat-Induced Aggregation. Wine samples were heated in a water bath at different temperatures (from 30 to 70 °C) and for different durations (from 5 to 360 min) before being cooled to room temperature. Precipitated proteins in wines following the different time−temperature heat-treatments were separated from the supernatant by centrifugation (20000*g*, 4 °C, 60 min). Proteins in the supernatants were analyzed by 1D-electrophoresis and their residual amounts were evaluated by image analysis, as described before. Each experiment was repeated twice.

DLS Experiments. DLS experiments were conducted with a Malvern Autosizer 3000 HS (Malvern Instruments, Malvern, UK) equipped with a 6 mW He−Ne laser (λ = 633 nm) and APD detection. Measurements were carried out at an angle of 90° from the incident beam. Prior to experiments, residual particles in wines were removed by centrifugation (3000*g*, 10 min.) and filtration on 0.2 μm microfiltration units (Millipore, Millex-GV). It was verified that microfiltration does not modify the wine composition in proteins. The sample (2−3 mL) was then introduced in the measurement cell, at a temperature of 25 °C. Control measurements were performed on wine samples before heating. The temperature was then increased to the heat treatment record value by heating with a Peltier device and maintained for 2 h. Following this heating step, the sample was cooled to 25 °C. Each experiment was done in duplicate. Sample evolution during heating and cooling was followed by measurements of the scattered intensity and of the autocorrelation function $G(t)$ of the scattered light. Each measurement was the average of 10 subruns. A rise in scattered intensity above that of the controls indicated the onset of aggregation.

The autocorrelation function of the scattered light was first analyzed using the cumulant method, which gives an average value of the aggregate size in the dispersion. The logarithm of the normalized correlation function is fitted to a polynomial

$$\log\left(\frac{G(t)}{B} - 1\right) = a + bt + ct^2 + ... \quad (1)$$

where $G(t)$ are the measured correlation points, B is the baseline, and a, b, and c are the coefficients of the cumulant fit determined by a simple linear least-squares fitting procedure (a is referred to as the "intercept", b the slope measures the "relaxation time" for the signal). The diffusion coefficient D of the colloids can be calculated as

$$\frac{1}{b} = 2DK^2 \quad (2)$$

where $K = (4\pi n_0/\lambda)\sin(\theta/2)$ is the scattering vector, n_0 is the solvent refractive index, λ is the laser wavelength, and θ is the scattering angle. The polydispersity index PI, defined as $c/2b$, measures the variance (standard deviation squared) of the distribution of particle size.

The average hydrodynamic diameters of the particles, D_h, are then derived from the diffusion coefficient D using the Stokes−Einstein equation and assuming spherical shapes

$$D = kT/(3\pi\eta D_h) \quad (3)$$

where k is the Boltzmann constant, T is the temperature, and η is the solvent viscosity. Results of DLS experiments obtained by the cumulant analysis provide the average hydrodynamic diameter D_h of the particles, in nm, and the polydispersity index PI of the suspension ($0 < PI < 1$).

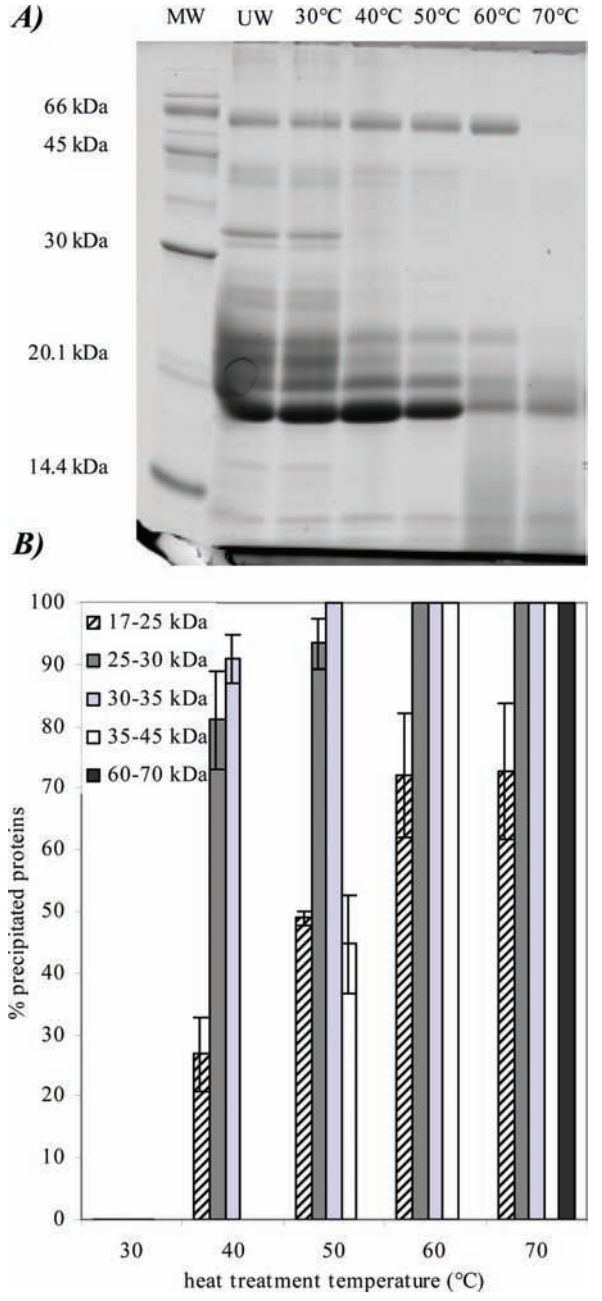

Figure 1. (**A**) 1D SDS−PAGE profile of the Sauvignon *Sa1* white wine (molecular weight MW standards on left) showing the different protein bands in the untreated wine (UW) and their depletion following to 2 h heat treatments at different temperatures (30, 40, 50, 60, and 70 °C). Untreated wine: most of the wine proteins (79.2%) were within the range 17−25 kDa. "Minor" bands were evidenced within the range 25−30 (8.3%), 30−35 (4.5%), 35−45 (3.5%), and 60−70 (4.5%) kDa. (**B**) Protein precipitation (expressed in %) in the Sauvignon *Sa1* wine following to 2 h heat-treatments at 30, 40, 50, 60, and 70 °C. Protein depletion was evaluated following to 24 h cooling at room temperature and aggregate removal by centrifugation.

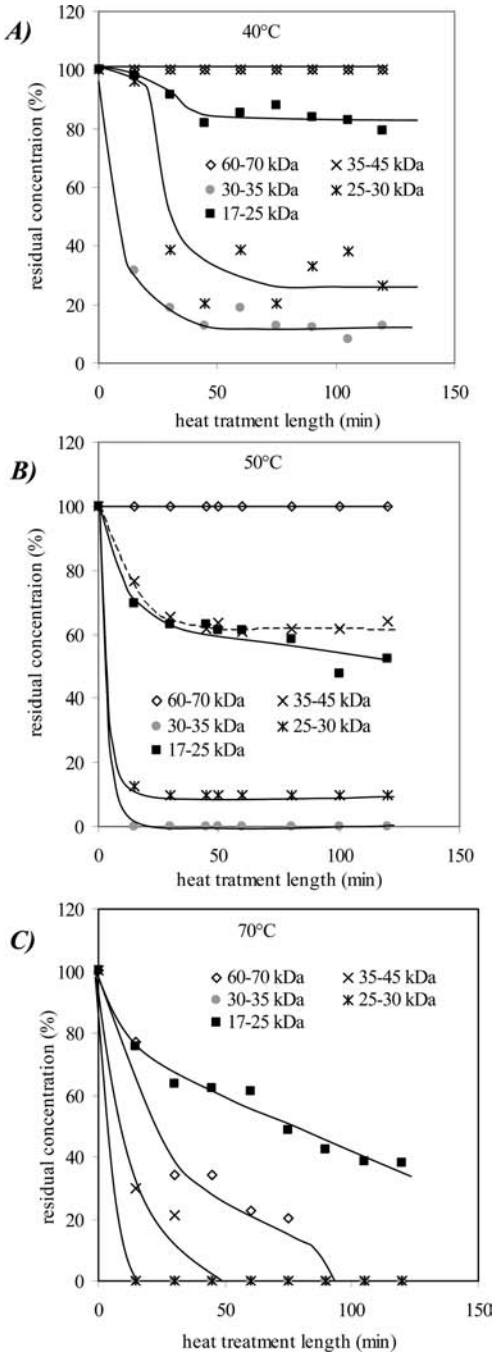

Figure 2. Protein depletion in the Sauvignon *Sa1* wine due to different heat-treatment durations at different temperatures. (**A**) 40 °C, (**B**) 50 °C, (**C**) 70 °C. Protein depletion was evaluated following 24 h cooling at room temperature and aggregate removal by centrifugation.

RESULTS AND DISCUSSION

Time−Temperature Dependence of Heat-Induced Protein Aggregation (Sauvignon *Sa1* Wine). The temperature impact on aggregation and aggregation kinetics was first studied with a Sauvignon wine, referred to as *Sa1* thereafter. The 1D SDS−PAGE protein pattern of this white wine was typical of those reported in the literature with a major protein band, usually assigned to thaumatin-like proteins, within the range 17−25 kDa (*28*). Other bands were evidenced within the ranges 25−30, 30−35, 35−45, and 60−70 kDa (**Figure 1A**). Proteins in wines are frequently identified using LC-MS/MS analyses of the tryptic peptides and database searching. The higher molecular weight proteins (64−66 kDa) are usually found to be invertases, whereas chitinases and β-glucanases are found within the range 26−32 and 35−42 kDa, respectively (*13*−*17*). As protein identification was not performed in the present study, the different proteins will be sorted according to their MW range, without assumptions concerning their identity.

Protein heat-induced precipitation following 2 h treatment at increasing temperatures (from 30 to 70 °C) and cooling was

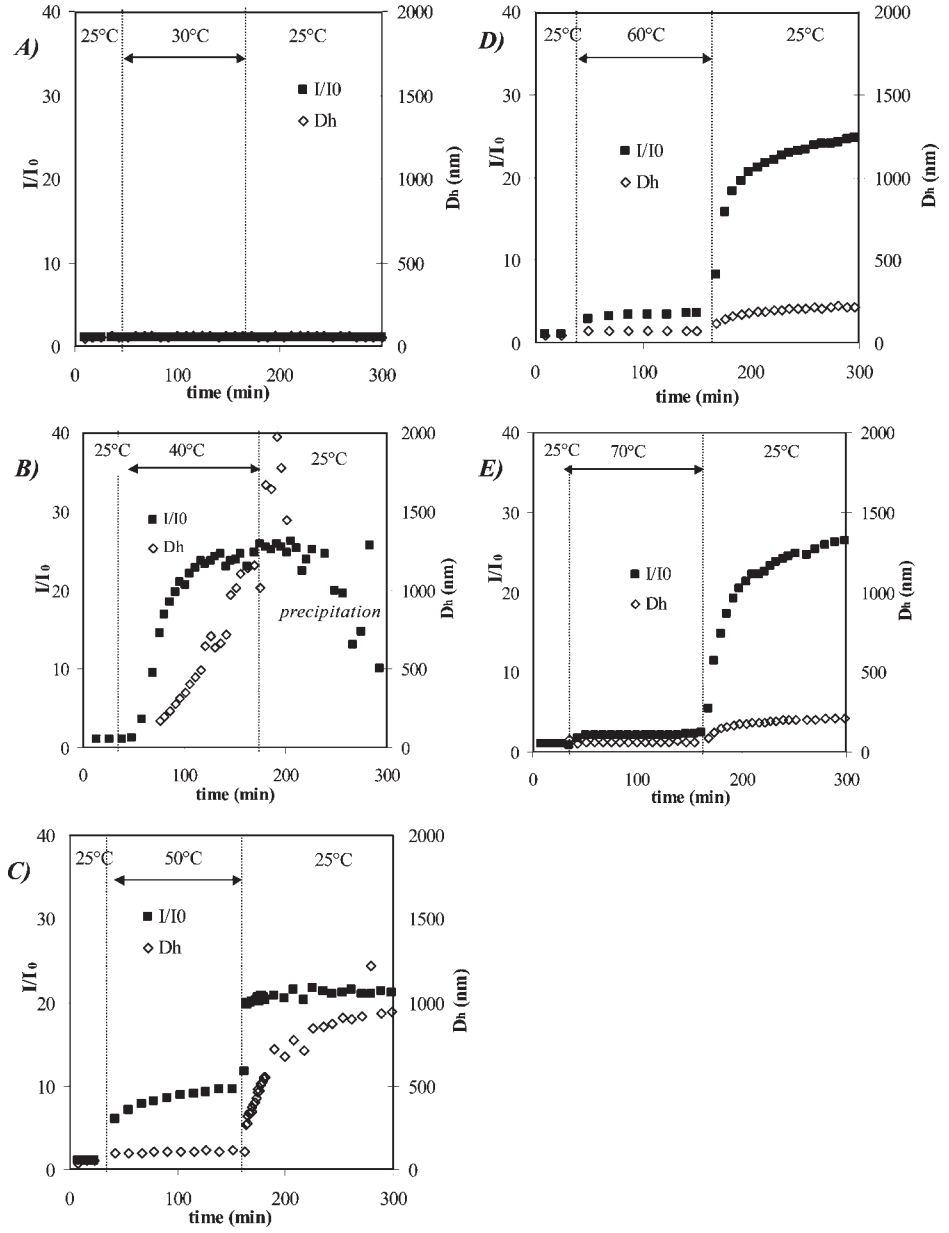

Figure 3. Aggregation kinetics in the *Sa1* wine at different temperatures followed by dynamic light scattering (observation angle 90°). (**A**) 30 °C, (**B**) 40 °C, (**C**) 50 °C, (**D**) 60 °C, (**E**) 70 °C. A rise in the normalized scattered intensity I/I_0 above 1 indicates the onset of aggregation (I_0 = intensity scattered by the wine before heating). D_h: average hydrodynamic diameter of the aggregates, weighted according to the intensity.

analyzed and quantified by 1D SDS−PAGE coupled with image analyses (**Figure 1A**,**B**). The different behaviors of wine proteins toward heat-induced precipitation were in accordance with previous data (*1*, *15*, *27*) and will be further discussed in the next sections. In addition, protein depletion was followed for different time−temperature heat treatments at 40, 50, and 70 °C (**Figure 2**). Submiting the wine at 30 °C during 2 h did not induce any detectable change in its protein composition (**Figure 1**). Heat-induced aggregation occurred when the temperature was augmented to 40 °C. After 2 h, most of the proteins within the range 25−30 and 30−35 kDa were heat-precipitated, along with some of the 17−25 kDa proteins. Time−temperature curves indicated that a plateau value for protein depletion was reached after about 1 h heating (**Figure 2A**). Increasing the temperature up to 50 °C increased protein depletion (**Figures 1** and **2B**). A plateau was reached within 30 min. Precipitated proteins belonged to the same molecular weight ranges than at 40 °C. In addition, part of the 35−45 kDa proteins were heat-precipitated. Precipitates formed following 2 h at 60 °C involved all the 25−30, 30−35, and 35−45 kDa proteins and significant amounts of the 17−25 kDa proteins. Those formed at 70 °C also involved the 60 kDa ones. At 70 °C, heat-induced depletion of the 25−30 and 30−35 kDa proteins was completed after 15 min, that of the 35−45 kDa after 60 min, and that of invertases after 90 min (**Figure 2C**). Residual proteins following 2 h at 70 °C were the lowest MW proteins within the range 17−25 kDa (**Figure 1A**).

Temperature Impact on Aggregation Kinetics and Aggregate Properties (Sauvignon *Sa1* Wine). Protein aggregation during wine heating and cooling was followed by DLS for the same heat-treatment temperatures (**Figure 3**). DLS experiments provided preliminary information concerning aggregation kinetics and mechanisms when wine was submitted to different temperatures.

Aggregation between macromolecules or particles in aqueous media is governed by a complex interplay between intermolecular Lifshitz-van der Waals, polar hydrogen donor/hydrogen

acceptor, and electrostatic interactions (*29*, *30*). The respective impact of these interactions depends on the physicochemical properties of the interacting species (charge, polarity) and of the suspending medium (pH, ionic strength, polarity). It also depends on the distance between these species and on their dimensions. According to the balance between these interactions, the net free energy of interaction ΔG may evolve as a function of the separation distance (d) in very different ways (extended D.L.V.O. theory) (*30*). The result can be (i) a strong attraction ($\Delta G(d) < 0$), leading to fast aggregation; (ii) a strong repulsion ($\Delta G(d) > 0$), leading to stability; (iii) a more complex interaction potential with the existence of a secondary minimum at a finite distance, of a more or less high energy barrier at smaller distances and of a strong primary minimum at very short distances. In this latter case, the stability of the system, and for unstable systems aggregation kinetics, will be dependent on the energy barrier and of the ability of the colloidal particles/macromolecules to overcome it.

The intensity scattered by the untreated wine (I_0) was low at 25 °C, with a very low signal-to-noise ratio (**Figure 3A**). Heat treatment at 30 °C did not induce any change of the scattering intensity, either during heating or cooling. This indicated that no detectable aggregation occurred during the experiment. As well, the wine protein composition was not affected (**Figure 1**). By contrast, a continuous rise of the relative scattering intensity (I/I_0) was observed as soon as the temperature was increased to 40 °C (**Figure 3B**). Aggregation led to the formation of highly polydisperse dispersions of particles (polydispersity index PI = 1) with large average hydrodynamic diameters (D_h). D_h thus represented the aggregate size weighted according to their scattering power, which is in favor of the largest particles in the dispersion. The average aggregate size kept increasing during the experiment, leading to the formation of micrometer-sized particles, whereas I/I_0 reached a plateau-value (I/I_0 in the order of 24) after about 90 min heating. This indicated that no new aggregates formed at this stage: particle growth was related to coaggregation of the previously formed aggregates. At 40 °C, interaction potentials in the *Sa1* wine were thus such that changes in protein conformations induced immediate attraction, leading to aggregation. When dealing with a complex medium such as wine, aggregation may not only involve unfolded/partly unfolded proteins, but also unaffected polypeptides and other wine components (*20*, *21*, *26*). This initial aggregation was immediately followed by aggregate growth due to the incorporation of new material and/or to attractive interactions between the aggregates. This hypothesis is supported by the effect of the 40 °C heat-treatment duration on protein aggregation (**Figure 2A**). Associated with DLS results this experiment allows one to follow, indirectly, the relationship between protein conformational changes and aggregation. A "plateau" value for protein depletion was reached after about 1 h heating, in agreement with the "plateau" value observed for the scattering intensity. Aggregate size kept increasing during the cooling step, leading to sedimentation (I/I_0 decreased) and to the formation of a cloudy haze characterized by light and large eye-visible flocs.

Increasing the temperature to 50 °C did not have a large impact on residual proteins (**Figures 1** and **2B**). However, DLS experiments showed the strong incidence of the temperature on colloidal equilibriums and aggregation mechanisms (**Figure 3C**). The onset of aggregation between species occurred from the very beginning of heating, as observed at 40 °C. It was evidenced by a rapid but moderate increase of I/I_0, followed by a much more progressive increase to a value of 9. This moderate increase of I/I_0 was related to the rapid formation of quite small "metastable" aggregates (D_h of 105 ± 5 nm, PI in the order of 0.4) the size of

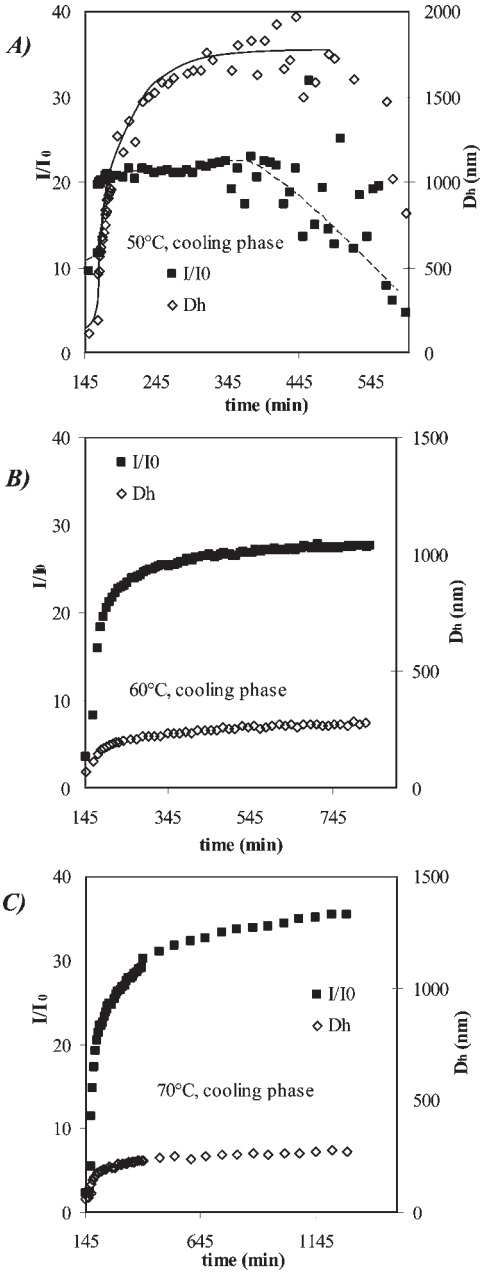

Figure 4. Details of the "long-term" aggregation kinetics observed at 25 °C following 2 h heat-treatments of the *Sa1* wine. (**A**) 50 °C, (**B**) 60 °C, (**C**) 70 °C.

which only slightly evolved during heating. Physicochemical interactions between these aggregates were thus such that a repulsion barrier at finite distance prevented their aggregation. This barrier vanished when the temperature was lowered, allowing aggregation of the primary aggregates into micrometer-sized particles. Enlarged aggregation (formation of micrometer-sized aggregates) occurred within the first minutes of the cooling phase. I/I_0 immediately rose up to 20 and stabilized, whereas the aggregate size kept increasing, indicating that micrometer-sized aggregates formed due to collision and sticking of smaller ones. It was followed by sedimentation after 3 h.

Increasing conformational changes, leading to enhanced aggregation, were expected and observed at 60 and 70 °C: analyses performed following to different heat-treatment lengths at 70 °C showed that changes in the protein conformations occurred from the very beginning of heating (**Figure 2C**). However, aggregation during heating was strongly prevented at 60 and 70 °C: I/I_0

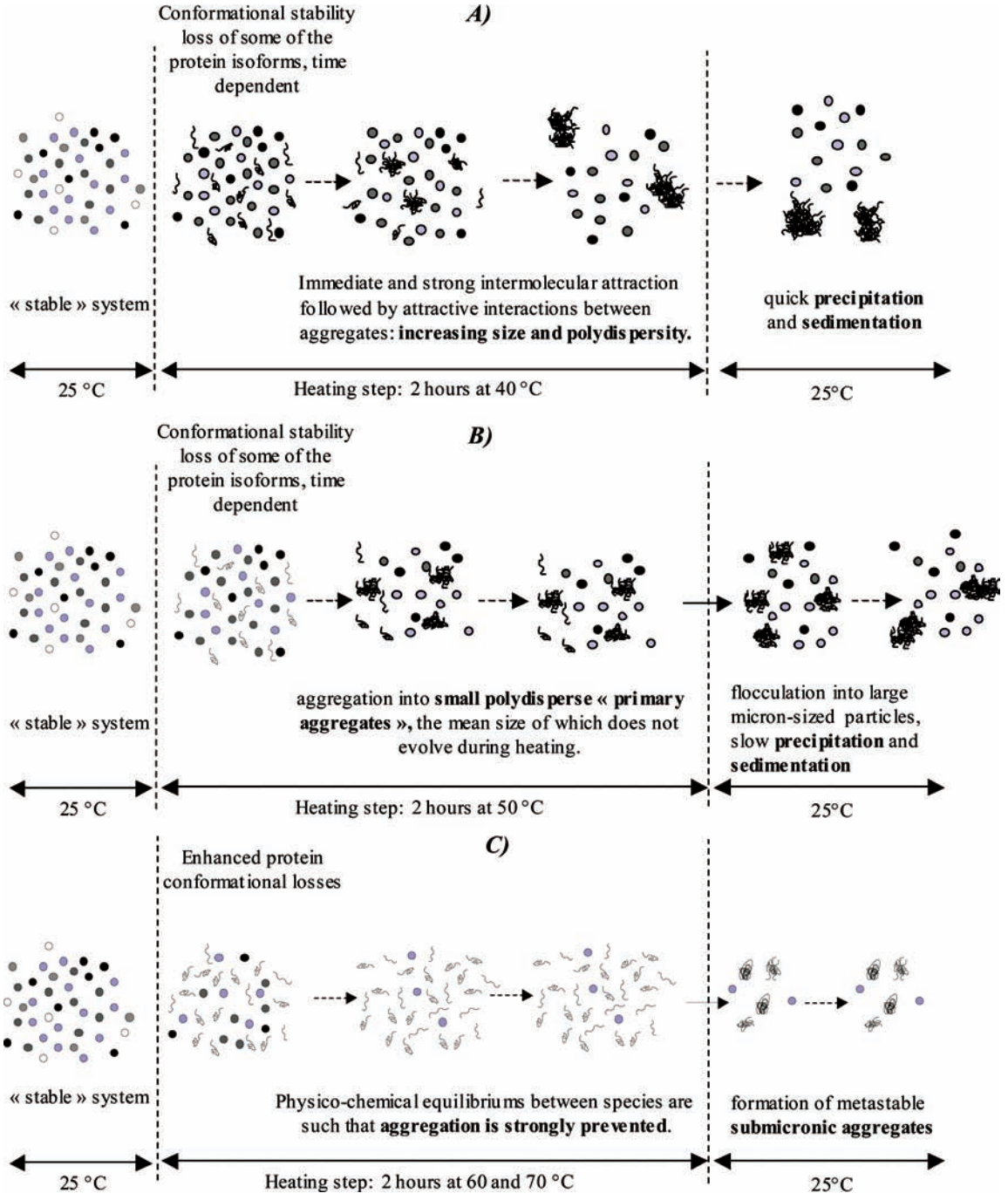

Figure 5. Schematic representation of protein aggregation in the Sauvignon *Sa1* during heating at increasing temperatures and cooling, illustrating the temperature impact on physicochemical equilibriums and aggregation kinetics. (**A**) 40 °C, (**B**) 50 °C, (**C**) 60 and 70 °C.

quickly stabilized to low plateau values (3.4 ± 0.2 and 2.4 ± 0.2 for 60 and 70 °C, respectively) and did not evolve (**Figure 3D**,**E**). Aggregation essentially occurred during cooling, leading to quite different dispersion characteristics than that observed after 40 and 50 °C heat-treatments. I/I_0 increased very quickly, but aggregates remained submicronic (average hydrodynamic diameter in the order of 270 nm) over a very long period. Details of the "long-term" aggregation kinetics observed during cooling for 50, 60, and 70 °C heat-treatments are shown in **Figure 4** for comparison. Aggregation was very slow at the ambient temperature, indicating very different physicochemical properties and thus very different structures of the aggregates by comparison to those formed at 40 and 50 °C. Visual observation after 24 h at room temperature showed the formation of a fine suspended haze in the wine that did not sediment. The different behaviors observed by DLS at the tested temperatures are summarized in **Figure 5**.

Additional experiments were performed to determine if the stability of the aggregates formed at high temperatures could be related to their charge, that is, to long-range electrostatic repulsions. Considering ionic contents in wines (*31*), the ionic strength mainly ranges between 10 to 100 mM. In such a range, the ionic strength strongly affects electrostatic interactions. From the concentration of its main cations (**Table 1**), the *Sa1* wine ionic strength can be estimated as being on the order of 20 mM (this value is likely underestimated). Salts (NaCl) were added to the 60 and 70 °C heat-treated wines during the cooling phase, so as to obtain final concentrations of 100 and 200 mM (**Figure 6**).

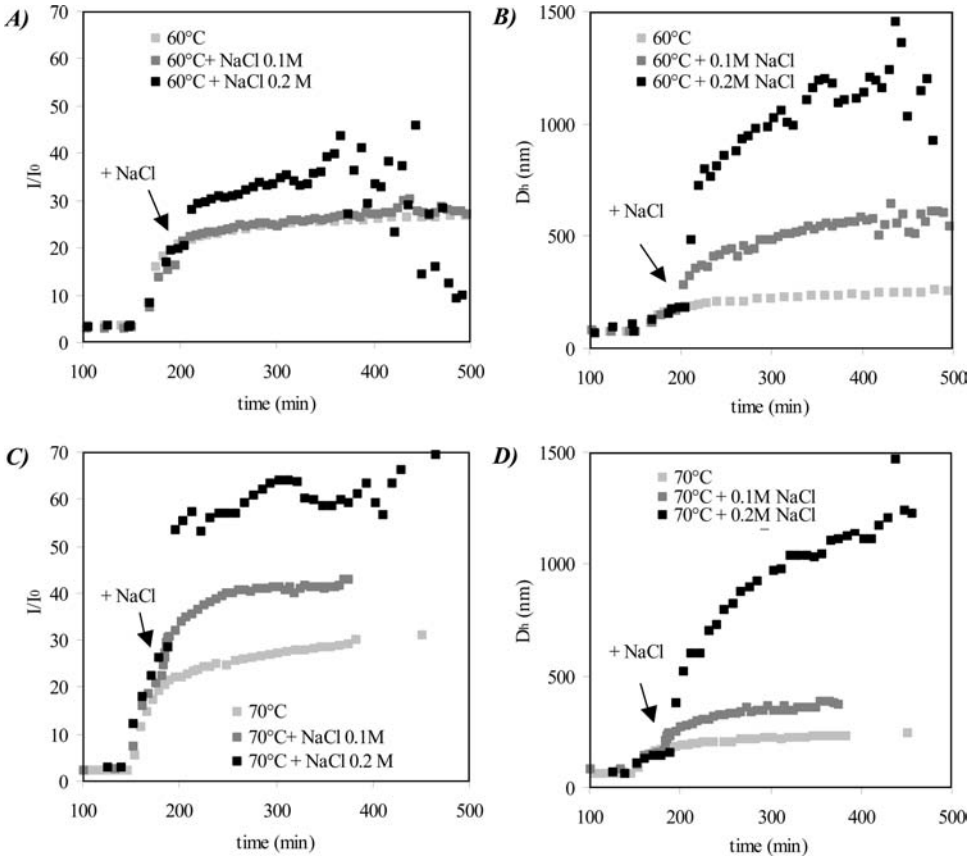

Figure 6. Impact of NaCl addition on the colloidal stability of the aggregates formed following to 2 h heat-treatments at 60 and 70 °C (*Sa1* wine). (**A**) 60 °C, relative scattering intensity I/I_0. (**B**) 60 °C, average hydrodynamic diameter D_h. (**C**) 70 °C, relative scattering intensity I/I_0. (**D**) 70 °C, average hydrodynamic diameter D_h.

Increasing the wine ionic strength by 100 mM led to an increase of the aggregate sizes, indicating that the repulsion barrier between the particles was lowered and that the probability for colliding particles to bond was increased. However, aggregation kinetics remained slow and no micrometer-sized aggregates formed during the experiment duration. Addition of 200 mM salts enhanced aggregation kinetics and large particles, prone to sedimentation, were formed within 1−2 h. Electrostatic repulsions thus played a determinant part in the colloidal stability of the aggregates formed following 60 and 70 °C heat treatments. The latter likely exhibit more charged surfaces than those formed at 40 and 50 °C. These differences may be linked to different physicochemical properties of the involved proteins, and support the hypothesis of coaggregation between the different isoforms.

Temperature Impact on Heat-Induced Protein Precipitation and Aggregation Kinetics: Comparison between Different Wines. DLS experiments, coupled with the study of the influence of different time−temperature treatments on protein aggregation, were repeated at 40 and 60 °C for another Sauvignon (*Sa2*) wine and for a Chardonnay (*Ch*) one. The aim was to compare aggregation kinetics and colloidal behaviors for different wines. The 1D SDS−PAGE profiles of these additional wines are given in **Figure 7**. For both wines, only few minutes' exposure at a temperature of 40 °C was enough to induce the aggregation of all the 30−35 kDa proteins (**Figure 8**). Increasing the heat-treatment length induced the precipitation of all the 25−30 kDa polypeptides and of a minor part (between 15 to 30%) of the major proteins (17−25 kDa). Protein depletion was mostly achieved within 1 h, as previously observed (**Figure 2A**). DLS results obtained at 40 °C (**Figure 8**) were also in accordance with those found with *Sa1* (**Figure 3B**): aggregation started as soon as the temperature was increased and further developed during heating. A "plateau" value for the scattering intensity was reached after about 1 h heating, in agreement with the "plateau" value for protein depletion. Particle growth at the end of the heating step and during cooling can then be essentially attributed to attractive interactions between the previously formed aggregates. When a temperature of 60 °C was applied (**Figure 9**), both the 25−30 and

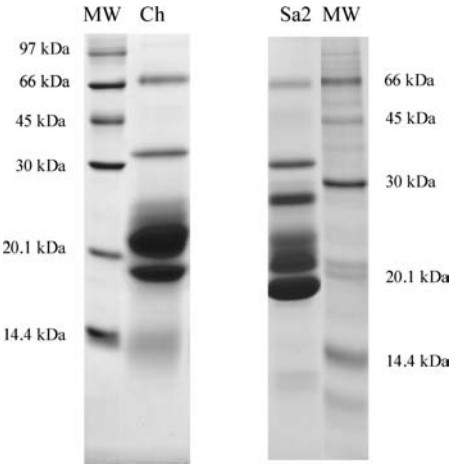

Figure 7. 1D SDS−PAGE profiles of the Sauvignon *Sa2* and Chardonnay *Ch* wines. MW: Molecular weight standards. Major proteins (17−25 kDa) represented 80.5 and 84.7% of the whole protein content in *Sa2* and *Ch*, respectively. 25−30 (9.9%), 30−35 (3.3%), and 60−70 (6.3%) kDa protein were also present in the *Sa2* wine, whereas only 30−35 (7.2%) and 60−70 (8.1%) kDa proteins were present in the Chardonnay.

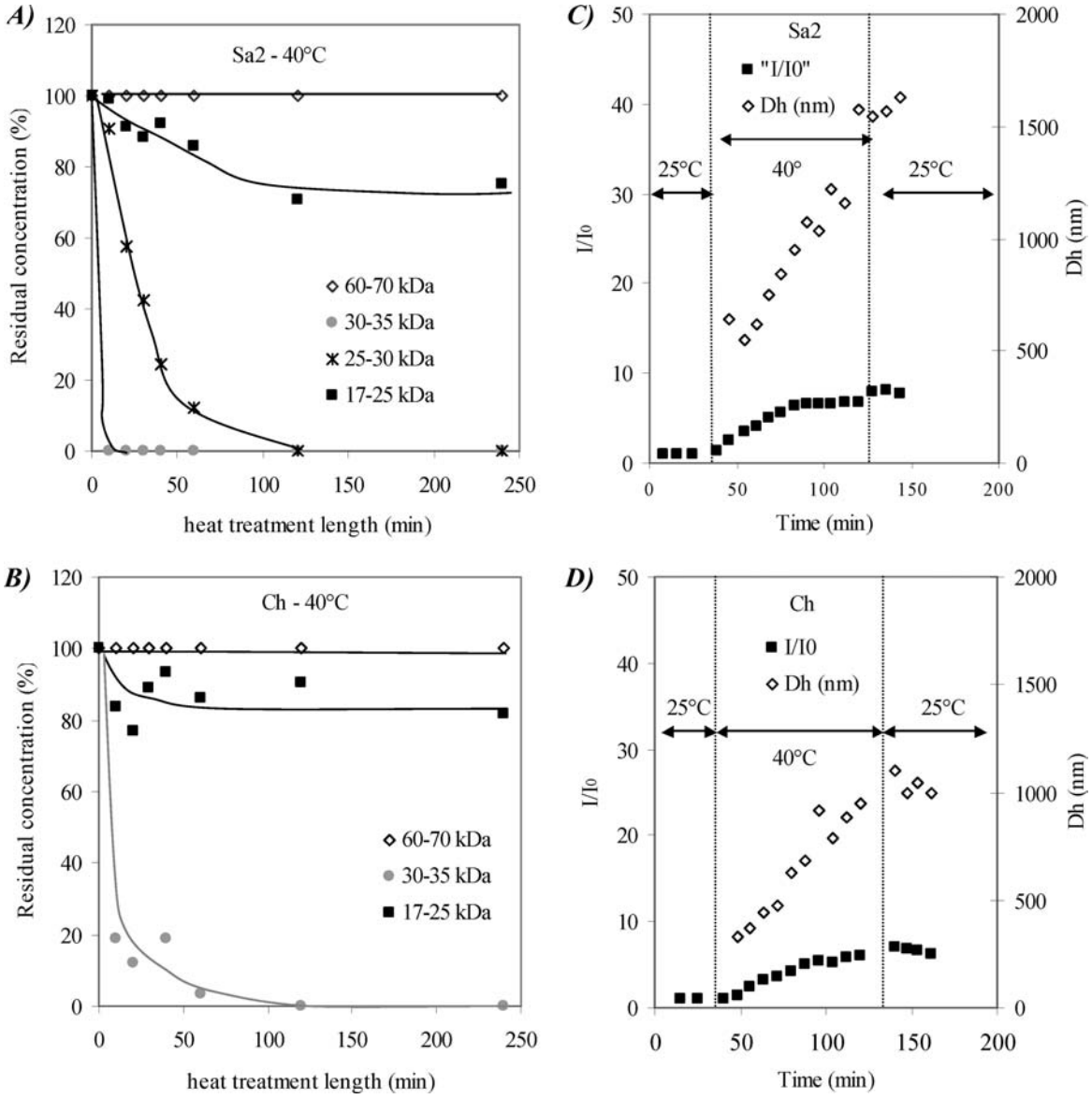

Figure 8. Left: protein depletion in the Sauvignon *Sa2* (**A**) and Chardonnay *Ch* (**B**) wines due to different heat treatment durations at 40 °C. Protein depletion was evaluated following 24 h cooling at room temperature and aggregate removal. Right: aggregation followed using DLS experiments (I/I_0: normalized scattering intensity of the sample, D_h: mean hydrodynamic diameter weighted according to the intensity). (**C**) Sauvignon *Sa2*. (**D**) Chardonnay *Ch*.

30−35 kDa proteins were fully precipitated from the lowest heat treatment lengths. Proteins in the size 60−70 kDa started to be affected. Heat-induced depletion of the 17−25 kDa proteins was enhanced and was faster and more important in the Chardonnay than in the Sauvignon *Sa2*. In accordance with that observed for *Sa1*, increasing the temperature strongly prevented protein aggregation during heating (**Figure 9**). Aggregation only started when the temperature was reduced. Temperature thus played for the three studied wines a determinant part in the colloidal stability of the unfolded proteins. The strong influence of the cooling phase suggests the involvement of polar interactions (H-bond formation) in the aggregation. Slow aggregation kinetics were observed with the *Sa2* wine, leading during the initial stages of cooling to the formation of metastable particles with finite average hydrodynamic diameters (D_h around 200 nm). In the Chardonnay *Ch*, aggregation quickly led to the development of micrometer-sized aggregates and precipitation. These different behaviors indicate different aggregate structures/properties and/or different colloidal equilibriums between the two Sauvignons on one hand and the Chardonnay on the other hand. These different behaviors, already reported in the literature, can neither be explained on only the basis of the wine protein compositions nor on the basis of the conventional enological parameters determined in the present study (pH, total polyphenols...). A possibility is the presence in the Chardonnay wine of a non-protein component required to induce the formation of haze upon heating (*21*).

Comparing protein depletion induced by the different heat treatments and in accordance with previous results (*1*, *15*), some trends can be deduced that are common to the three studied wines. These trends can be compared with the recent results of Falconer et al. (*27*), who studied the thermal stability of thaumatin, chitinase, and invertase isoforms purified from grape juices by differential scanning calorimetry. Temperatures as high as 60 °C are generally needed to provoke some heat-induced precipitation of the 60−70 kDa proteins, which remains moderate unless 70−80 °C treatments are applied. This protein class, usually assigned to invertases, can thus be considered as heat-resistant and will not be primarily involved in haze developments during wine transport and storage. Accordingly, a melt temperature of

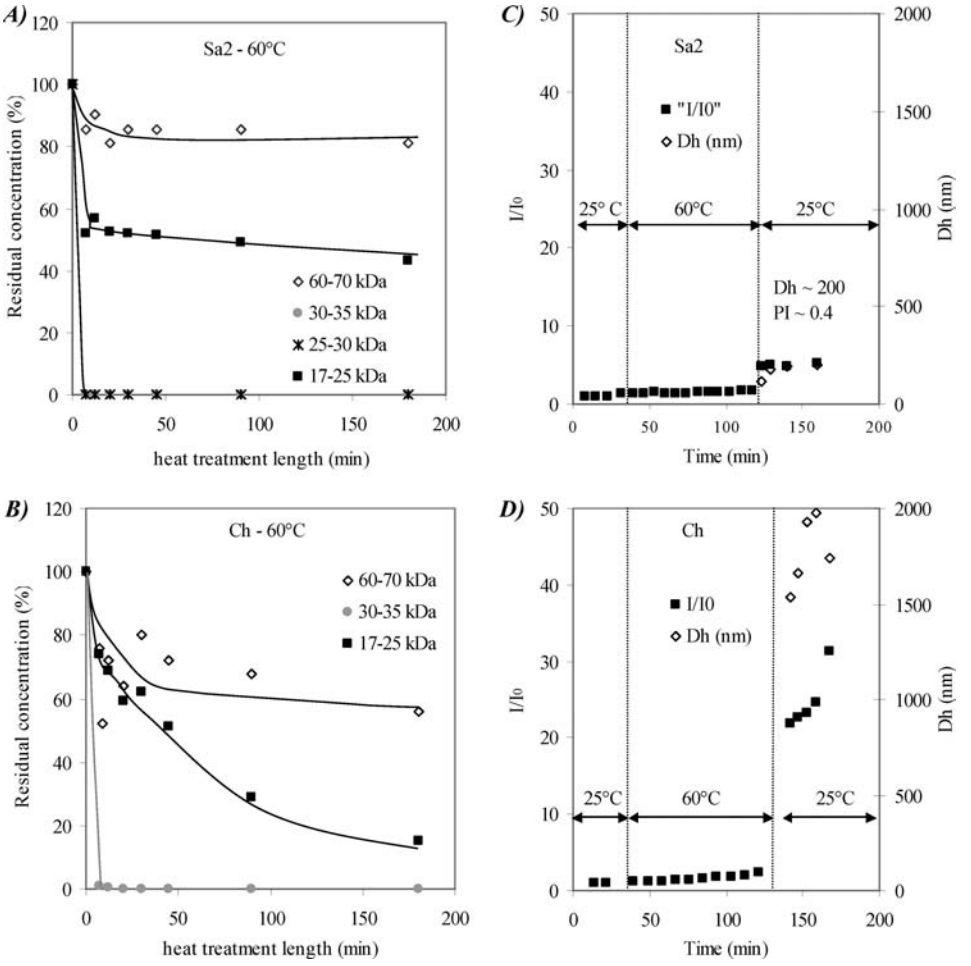

Figure 9. Left: protein depletion in the Sauvignon *Sa2* (**A**) and Chardonnay *Ch* (**B**) wines due to different heat treatment durations at 60 °C. Protein depletion was evaluated following 24 h cooling at room temperature and aggregate removal. Right: aggregation followed using DLS experiments (I/I_0: normalized scattering intensity of the sample, D_h: mean hydrodynamic diameter weighted according to the intensity). (**C**) Sauvignon *Sa2*. (**D**) Chardonnay *Ch*.

81 °C was found for a Sauvignon blanc juice invertase (*27*). It can be noted that the present results show that heat-induced precipitation can occur for temperatures well below that melting point, which was determined in a model wine. Moreover, invertase did not aggregate following heating at 80 °C in that model wine, in contradiction with results found in these wines. This may indicate an impact of the wine matrix on protein thermal stability, or an aggregation/coaggregation of the partially unfolded proteins in a more complex system. By contrast, only short exposures to moderate temperatures (40 °C) were enough to induce the aggregation of some of the proteins and to induce visible haze. This especially heat-sensitive behavior concerned proteins within the ranges 25−30 and 30−35 kDa, usually assigned to chitinases and β-glucanases. β-Glucanases were previously shown to be the most heat-unstable proteins in a Chardonnay wine: they were fully precipitated by a 30 min 40 °C heat-treatment (*15*). The study of the thermal stability of Class IV chitinases showed that these proteins were the least stable when compared to invertases and thaumatins (*27*). Accordingly, these proteins were previously shown to be strongly affected by short-time (30 min) low temperatures (40 and 50 °C) heat treatments (*15*). It is worth noting that the most important variability toward heat-induced precipitation was found for the 17−25 kDa proteins that are the most abundant in wines and usually assigned to thaumatins. These proteins exhibited very different heat-sensitivity within one and a same wine and between different wines (**Figures 1**, **2**, **8**, and **9**). Some were very heat-sensitive, whereas others resisted 2 h heat treatments at high temperatures. These differences may have different causes: (i) different protein classes within the same MW range and/or different protein isoforms with different conformational stabilities and physicochemical behaviors (*11*, *12*, *17*, *26*, *32*); (ii) different wine compositions leading to different matrixes (ethanol, pH, ionic strength and ionic contents) and/or cosolute contents (polyphenols, polysaccharides, ...) and thus to different behaviors with regard to heat-induced unfolding and aggregation.

Heat-forced aggregation is widely used to accelerate phenomena. It is used both to assess the risks of instability development but also to investigate the involved physicochemical mechanisms. However and according to current knowledge concerning protein aggregation, which has been the subject of numerous research in different fields, these physicochemical mechanisms are temperature-dependent. It can then not be stated that results obtained from high temperature treatments reflect the conformational changes and colloidal phenomena that would occur if low-temperature long-time conditions were applied. DLS results clearly show that temperature affects not only protein contents but also aggregation mechanisms and that this parameter is to be accounted for to identify the latter. Comparison between the Chardonnay and the Sauvignon wines also underlined that physicochemical mechanisms are likely wine-dependent, in accordance with previous observations. Are the different behaviors observed between these wines following heat-treatment at 60 °C related to different protein isoforms? To different matrixes (pH, ionic strength, type of ions, cosolutes, ...)? The two phenomena

involved in haze formation, protein unfolding, and colloidal interactions, were not treated separately here. This, along with the variability of the wine composition, hinders the interpretation of the data and the identification of the mechanisms involved in haze formation. Further studies will have to be managed in order to distinguish between protein conformational changes and colloidal equilibriums in wines. This involves the purification of wine proteins and the comparison of studies in both model and real conditions. Present results underline that DLS experiments, coupled with the study of protein thermal stability, can be a powerful tool to determine the influence of wine characteristics and composition on protein colloidal stability.

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