JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

# Effects of Ionic Strength and Sulfate upon Thermal Aggregation of Grape Chitinases and Thaumatin-like Proteins in a Model System

Matteo Marangon,[†] Francois-Xavier Sauvage,[§] Elizabeth J. Waters,[†] and Aude Vernhet*[,#]

[†]The Australian Wine Research Institute, P.O. Box 197, Glen Osmond (Adelaide), SA 5064, Australia

[§]INRA, UMR1083 Sciences Pour l'Oenologie, 2 place Viala, F-34060 Montpellier, France

[#]Montpellier SupAgro, UMR1083 Sciences Pour l'Oenologie, 2 place Viala, F-34060 Montpellier, France

Supporting Information

**ABSTRACT:** Consumers expect white wines to be clear. During the storage of wines, grape proteins can aggregate to form haze. These proteins, particularly chitinases and thaumatin-like proteins (TL-proteins), need to be removed, and this is done through adsorption by bentonite, an effective but inefficient wine-processing step. Alternative processes are sought, but, for them to be successful, an in-depth understanding of the causes of protein hazing is required. This study investigated the role played by ionic strength ($I$) and sulfate toward the aggregation of TL-proteins and chitinases upon heating. Purified proteins were dissolved in model wine and analyzed by dynamic light scattering (DLS). The effect of $I$ on protein aggregation was investigated within the range from 2 to 500 mM/L. For chitinases, aggregation occurred during heating with $I$ values of 100 and 500 mM/L, depending on the isoform. This aggregation immediately led to the formation of large particles (3 $\mu$m, visible haze after cooling). TL-protein aggregation was observed only with $I$ of 500 mM/L; it mainly developed during cooling and led to the formation of finite aggregates (400 nm) that remained invisible. With sulfate in the medium chitinases formed visible haze immediately when heat was applied, whereas TL-proteins aggregated during cooling but not into particles large enough to be visible to the naked eye. The data show that the aggregation mechanisms of TL-proteins and chitinases are different and are influenced by the ionic strength and ionic content of the model wine. Under the conditions used in this study, chitinases were more prone to precipitate and form haze than TL-proteins.

**KEYWORDS:** protein aggregation, thaumatin-like protein, chitinases, haze, wine, grape juice, ionic strength, sulfate

## INTRODUCTION

To meet consumers' expectations, white wines always need to be brilliantly clear. However, bottled white wines can turn hazy and form sediments. Research and experience have led to the understanding that haze formation in white wine is associated with the presence of residual grape proteins[1] and with elevated temperatures during storage or transportation. Grape proteins in wines, being unstable under certain conditions, can aggregate into light-dispersing particles to make wines appear turbid.[2−4] Consequently, these proteins, and particularly chitinases and thaumatin-like proteins (TL-proteins),[5,6] need to be removed before bottling. Protein removal is achieved by fining wines with bentonite, a clay negatively charged at wine pH, that binds to the positively charged wine proteins and settles to the bottom of the tanks. The use of bentonite was introduced in 1934[7] and is still widely used, despite having many drawbacks. Bentonite strengths are its low cost, its availability, and its efficacy in removing proteins and, thus, in stabilizing wines. However, its use is generally associated with several disadvantages and direct or indirect costs, such as loss of wine volume (3−10%) due to poor settling, environmental costs for bentonite disposal because it is not reusable, and labor costs for its application.[8] Therefore, alternatives are sought.

So far, the search for alternatives to bentonite has not yet resulted in commercially viable solutions able to compete with bentonite's efficacy and low cost. It is believed that a better comprehension of the causes for haze is needed to succeed, because a thorough understanding of the mechanisms of protein haze formation has the potential to lead to the development of novel, efficient, and environmentally sustainable winemaking processes to prevent haze from forming.

It is generally thought that grape proteins denature and then aggregate together and that the size of the aggregated protein particles, and thus their visual presence, depends on other wine solutes such as sulfate, polyphenols, and polysaccharides[9−11] Despite their strong impact on protein stability, there have been only a few studies focused on the effects of wine pH and salts (ionic strength).[12,13] The identity and relative importance of pH and ionic strength in haze formation are yet to be fully understood.

Salts affect the stability of proteins by modifying the ionic strength of the solution. Their overall effect on protein conformation can be both stabilizing and destabilizing,[14,15] depending on the nature of the specific charge distribution within the protein.[16] Furthermore, for a pure protein system, protein−protein interactions are generally favored at conditions that reduce the net charge on the molecules, that is, at pH values close to the isoelectric point[17] or at high ionic strengths.[18] On the basis of their usual ionic contents, normal wine ionic strength ranges between 10 and 100 mM.[19] In such a range, we would expect differences in ionic strength to strongly influence

Received: November 8, 2010
Revised: January 23, 2011
Accepted: February 1, 2011
Published: March 01, 2011

**Table 1. Characteristics of Purified Proteins**

| protein name | protein type | same as[a] | $T_m$[b] (°C) | measured mass[c] | theoretical MW | theoretical pI | theoretical protein charge at pH 3[d] | NaCl concentration for SCX elution[e] (mM/L) |
|---|---|---|---|---|---|---|---|---|
| M1 | class IV chitinase | A | 55 | 25634 | 25617 | 5.15 | 14.4 | 71 |
| M2 | TL-protein | B | 61 | 21262 | 21276 | 4.54 | 10.5 | 71 |
| N | putative TL-protein | C | 61 | 21248 | 21240 | 4.76 | 15.1 | 150 |
| O | class IV chitinase | D1 | 55 | 25946 | 34680 | 7.47 | 22.8 | 250 |

[a] Names from Van Sluyter et al.[24] [b] Data from Falconer et al.[29] [c] Intact protein masses were determined by ESI-MS. [d] Measured via protein charge calculation tool at http://vitalonic.narod.ru/biochem/index_en.html. [e] Extrapolated from Supporting Information Figure S1A.

electrostatic interactions.[20,21] Moreover, different mechanisms may be involved depending on the ion type.[18,22] For instance, a recent study indicated sulfate anion as a candidate for the missing essential factor required for haze formation.[9]

The aim of the present study was to assess the role played by ionic strength and sulfate toward the aggregation of thaumatin-like proteins and chitinases upon heating. Wine haze formation is commonly studied by quantifying the turbidity produced by grape proteins upon heating,[10] a practice (named "heat test") used for the prediction of wine stability since the 1970s.[23] However, measuring the amount of haze after a heat test provides information about only the final step and not the early stages of protein aggregation. Therefore, in the present study heat tests were coupled with dynamic light scattering (DLS) experiments to follow the early stages of protein aggregation as well as the final step.

## MATERIALS AND METHODS

**Materials.** The model wine used in all experiments was prepared with 12% ethanol and 4 g/L tartaric acid and buffered to pH 3.0 with KOH. The ionic strength of the model wine was 2 mM.

**Purification of Proteins.** Chitinases and TL-proteins were isolated from 7.5 L of a 2005 Semillon grape juice sourced from South Australia. Proteins were purified by strong cation exchange (SCX) and hydrophobic interaction chromatography (HIC) as described by Van Sluyter et al.[24] (for more details see Figure S1 of the Supporting Information). The purity and identity of collected fractions were assessed by SDS-PAGE, RP-HPLC, ESI-MS/MS (Table 1 and Supporting Information Figure S2) and comparison with results obtained previously.[24] Proteins were stored as ammonium sulfate suspensions at 4 °C.

**Protein Preparation.** Upon utilization, proteins were prepared as follows: ammonium sulfate suspensions were centrifuged (13000*g*, 15 min, 4 °C), and the pellet was dissolved in ultrapure water. Salt removal and protein concentration were achieved via centrifugation with Nanosep 3 K ultrafiltration devices (Pall Corp., Glen Cove, NY). Concentrated proteins were held at 4 °C in model wine for a maximum period of 4 weeks. These stock solutions were then further diluted in the appropriate medium for heat tests and DLS measurements.

**Protein Content Determination.** Protein content was determined either by UV absorbance[25] at 280 nm (extinction coefficient calculated via http://ca.expasy.org/tools/protparam.html) or by EZQ protein quantitation kit (Invitrogen, Mt. Waverley, VIC, Australia) following the manufacturer's instructions. The calibration curve was built using serial dilution from 0 to 250 mg/L of thaumatin from *Thaumatococcus daniellii* (Sigma-Aldrich, Castle Hill, NSW, Australia). Fluorescence measurements were taken using excitation/emission settings of 450/618 nm with a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA).

**Protein HPLC.** The purity of proteins was determined by reverse-phase HPLC with a Vydac 2.1 × 250 mm C8 column (208TP52, Grace Davison Discovery Sciences, Baulkham Hills, NSW, Australia) on an Agilent Technologies 1200 system (Santa Clara, CA) according to the method of Marangon et al.[26] with modifications as suggested by Van Sluyter et al.[24] Injection volumes were 25 μL. From the 210 nm chromatogram, protein identity was assigned by comparison with the retention times of purified grape PR proteins.[24,26]

**Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE).** SDS-PAGE was performed with NuPage 10% Bis-tris, 1.5 mm thick, 10 well gels (Invitrogen) and an XCell SureLock Mini Cell (Invitrogen) following the manufacturer's instructions. Approximately 50 mg of $Na_2S_2O_5$ was added to the top reservoir prior to running to prevent cysteine oxidation. Samples were prepared by dissolving 3 μg of purified proteins in 20 μL of loading buffer (Invitrogen NuPage recipe) with 5% 2-mercaptoethanol. Precision Plus Protein unstained standards were from Bio-Rad laboratories Pty Ltd. (Regents Park, NSW, Australia). Proteins were stained with Pierce Imperial Protein Stain (Quantum Scientific, Sydney, NSW, Australia) according to the manufacturer's microwave instructions.

**Protein Electrospray Ionization Mass Spectrometry (ESI-MS).** Masses of purified proteins were determined according to the method of Hayasaka et al.[27] with modifications as described by Van Sluyter et al.[24]

**Heat Test Conditions.** Samples were heated at 70 °C for 2 h and cooled at 25 °C. After 20 h, the haze was measured by calculating the difference in the absorbance values at 540 nm[28] between the heated and unheated samples. Formed aggregates were separated by centrifugation (21000*g*, 15 min, 15 °C), and protein content was measured on supernatants.

**DLS Measurement.** DLS experiments were performed using a Malvern Zetasizer 3000 HS apparatus (Malvern Instruments, Malvern, U.K.), equipped with a 10 mW He−Ne laser (λ of 633 nm) and APD detection. Measurements occurred at 90° from the incident beam and were performed on samples while heat-tested as follows: 30 min at 25 °C (blank), 70 °C for 2 h (heating), 25 °C for variable length depending on the kinetics of aggregation (cooling). Five minutes was required to bring the sample to 70 °C (heating), whereas 8 min was needed to decrease the temperature to 25 °C (cooling). The temperature was controlled by a Peltier device. Sample evolution during heating and cooling was followed through measurements of the scattered intensity and the autocorrelation function of the scattered light. Each measurement was the average of 10 subruns. The average diffusion coefficient, *D*, of the scattering particles was extracted from the analysis of the autocorrelation function by the cumulant method. The average hydrodynamic diameter of the particles, Dh, was then derived from the diffusion coefficient using the Stokes−Einstein equation and assuming spherical shapes:

$$D = kT/(3\pi\eta \mathrm{Dh}) \quad (1)$$

*k* is the Boltzmann constant, *T* the absolute temperature, and η the solvent viscosity. Analysis of the autocorrelation function by the

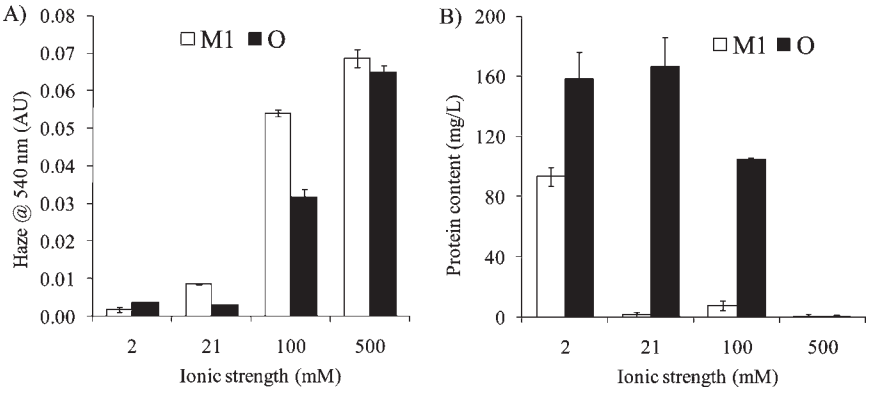


**Figure 1.** Chitinases were dissolved in model wine containing increasing dosages of NaCl to obtain *I* levels of 2 mM (no salt), 21 mM (1.23 g/L NaCl), 100 mM (5.85 g/L NaCl), and 500 mM (29.25 g/L NaCl): (A) haze (at 540 nm) of samples after heat test (analyses were performed after samples were cooled for 20 h at 25 °C); (B) protein content (measured by EZQ) in the supernatant obtained from centrifugation of samples (21000*g*, 15 min, 15 °C) after heat test. Protein contents in the untreated samples were 93.2 ± 12.4 and 158.2 ± 35.8 mg/L for M1 and O, respectively.

cumulant method also provides the polydispersity index PI of the suspension (0 < PI < 1). It is important to note that, for polydisperse suspensions (PI > 0.3), cumulant analysis gives a Dh value weighted according to the scattering intensity, which is in favor of the largest particles in the suspension.

To normalize the data of scattering intensity among samples, values were expressed as the ratio ($I/I_0$) between the scattering intensity during the analysis ($I$) and that of the blank ($I_0$). All assays were made in duplicate or triplicate on independently prepared solutions. The haze of samples after analysis was measured spectrophotometrically (540 nm absorbance). Formed aggregates were separated by centrifugation (21000*g*, 15 min, 15 °C), and protein content was measured on supernatants.

**Sample Preparation.** Each sample analyzed by heat test and DLS was prepared by diluting the concentrated stocks of proteins in the model wine at a wine-like concentration (≈100 mg/L). The initial concentration in the stock solution was estimated from the UV absorbance method. The dilution factor was adjusted to reach a final protein concentration on the order of 100 mg/L. Protein depletion in the samples due to heat treatments was estimated from the EZQ method. Discrepancies in absolute values for protein quantification are attributable to differences in methods used. Concentrated NaCl (50 g/L) or $Na_2SO_4$ (50 g/L, sulfate represent 67.6% of this entity) was used to adjust to the desired ionic strength in the samples. Each stock solution was filtered (0.22 $\mu$m). Final samples were centrifuged (13000*g*, 10 min, 10 °C) prior to DLS measurements.

## RESULTS AND DISCUSSION

**Impact of Ionic Strength on the Aggregation of Chitinases.** The incidence of ionic strength on protein aggregation was first investigated by a heat test. Two chitinase isoforms (M1 and O) were tested with *I* ranging between 2 and 500 mM. Heat treatments performed at low *I* (2 mM) neither affected the protein concentration nor induced haze formation (Figure 1). M1 fully precipitated at *I* of 21 mM and higher. In terms of haze, M1 gave little turbidity at *I* of 21 mM and much higher and visible haze with *I* of 100 or 500 mM. Protein O acted differently, its solubility being apparently unaffected by the heat test at *I* of 21 mM. O started to precipitate with higher *I* (100 mM) as shown by the appearance of turbidity (Figure 1A) and by the reduction of protein concentration in solution (Figure 1B); its precipitation was complete at *I* = 500 mM .

The data shown in Figure 1 are only a measurement of the effects of heat on protein solubility but do not give information about how and which aggregates were formed. To attain such data, the aggregation of proteins M1 and O was followed by DLS (Figure 2). When proteins were added to model wine without additional salt (*I* = 2 mM), the heating/cooling cycle affected the scattering intensity but at a very low extent. For M1 (Figure 2A) $I/I_0$ passed from 1 to 3 during heating and kept increasing slowly throughout the cooling (up to 7−8). This increase indicates that some modifications occurred. Because the melting temperature of M1 is 55 °C (see Table 1), 2 h at 70 °C was sufficient to promote protein unfolding; this unfolding could account for the observed $I/I_0$ increase. Because of the high polydispersity of the sample (polydispersity index, PI, = 1), the size could not be assessed with certainty, even if aggregation into very small particles was detected (Dh ≈ 50 nm, Figure 2B). The sample remained stable and did not evolve to form visible turbidity, confirming the data shown in Figure 1. At *I* of 21 mM, a slow but significant increase in the $I/I_0$ value (from 2 to 56 in 500 min) was observed as soon as the temperature was lowered to 25 °C (Figure 2A), indicating that M1 started to aggregate. The aggregation went on very slowly, leading to the formation of metastable aggregates with a Dh of 120 nm after 500 min (Figure 2B) and a Dh of 200 nm after 2 days (end of the experiment, not shown). At the end of the experiment $I/I_0$ had reached a plateau value of 80, but no haze was visible to the naked eye. After centrifugation, the supernatant was almost protein-free, demonstrating that most of the proteins initially present were involved. Increasing *I* to 100 mM caused the quick aggregation of M1 into large micrometer-sized aggregates (inset in Figure 2B), prone to sedimentation. Sedimentation was evidenced by the rapid decrease of the relative scattered intensity. Contrary to what was observed before, with this *I* aggregation took place as soon as the heat was applied. In this case the haze was visible and measurable (Figures 1A and 2B, inset) and protein precipitation almost complete (Figure 1B). The same behavior was observed with *I* at 500 mM (Figures 1 and 2A,B).

The second chitinase tested, O, behaved differently. No aggregation could be detected at *I* of 2 and 21 mM (Figure 2C, D). *I* of 100 mM induced aggregation that could be detected only during cooling (Figure 2C). The kinetics of aggregation was slow and showed a measured increase of both $I/I_0$ and Dh

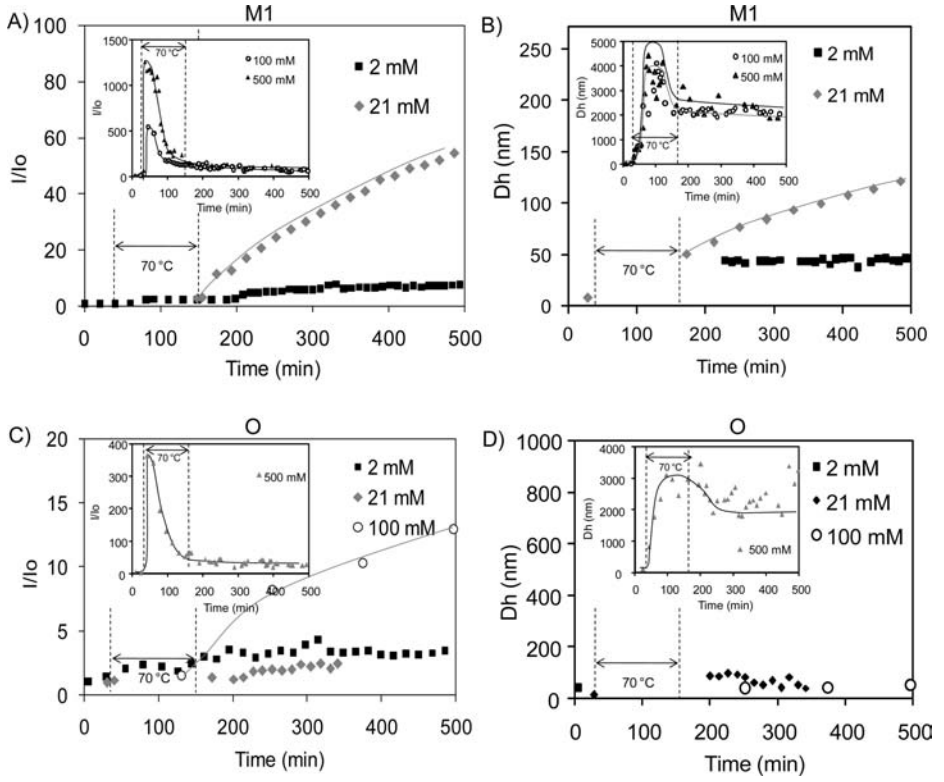


**Figure 2.** DLS measurements of proteins M1 and O with increasing *I*: (A) effect of ionic strength on normalized scattering intensity ($I/I_0$) for M1; (B) particle size (Dh) of M1 at increasing *I*: (C) effect of ionic strength on $I/I_0$ for O; (D) particle size (Dh) of O at increasing *I*. The protein content before the analysis was measured by the UV method and was between 81 and 88 mg/L for M1, and between 83 and 85 mg/L for O. Curves from the samples at high *I* are shown in the insets because their scale was too different from that of low *I* samples to be represented in the same graph. For chitinase O at 100 mM *I* the aggregation kinetics occurred progressively, reaching after 3 days a $I/I_0$ value of 80 with aggregates having an average Dh of 140 nm.

(Figure 2C,D). After 3 days $I/I_0$ was on the order of 80 and the average size of aggregates was ≈140 nm (not shown). These data were in agreement with the protein quantification (Figure 1B), which showed some protein depletion (34%) following heat treatment. This change gave measurable (Figure 1A) but not visible turbidity to the naked eye. With *I* of 500 mM O (insets in Figure 2C,D) behaved exactly as M1 did for *I* of 100 mM: large aggregation occurred as soon as the heat was applied with formation of visible haze.

Increasing ionic strength may affect both protein conformational stability (melting temperature) and colloidal stability. Chitinases at low *I* are denatured at temperatures around 55 °C (see Table 1). Therefore, in our conditions, we assumed that heating the proteins at 70 °C for 2 h was sufficient to cause their unfolding[29] and that *I* mainly affected colloidal stability. Colloidal aggregation of macromolecules (particles) in aqueous media results from a complex interplay between Lifshitz−van der Waals, polar hydrogen donor/hydrogen acceptor (hydrogen-bonding), and electrostatic interactions.[18,21] These interactions are temperature dependent, and their respective impacts depend on the physicochemical properties of the interacting species (charge, polarity) and of the suspending medium (pH, ionic strength, polarity). The impact also depends on the distance between these species and their dimensions. Lifshitz−van der Waals forces and H-bonding develop between the solvent molecules (solvent cohesion), between solvent molecules and macromolecules (particles), and between macromolecules to different extents depending on their respective properties. Lifshitz−van der Waals forces between biological macromolecules immersed in aqueous media are usually attractive and small, and the total interaction is mainly driven by polar and electrostatic forces.[21] Hydrogen-bonding interactions between two macromolecules (particles) in water can be attractive, leading to so-called "hydrophobic interactions", or repulsive, leading to "hydrophilic repulsion" or hydration pressure. Electrostatic interactions between species carrying the same charge are repulsive. These interactions are related to the overlapping of the electrical double layer that surrounds charged macromolecules/particles in solution. Their importance and rate of decay with distance is affected by the macromolecule/particle charge as well as by the solvent ionic strength. An increasing ionic strength, by decreasing the ionic double-layer thickness, reduces electrostatic interactions (repulsion in the case of species that carry the same charge), thus favoring their aggregation unless other stabilizing forces are present.

On the whole (as summarized in Figure 3) the results show that heat-induced aggregation/precipitation of chitinases was strongly influenced by ionic strength, highlighting the impact of electrostatic repulsions on their colloidal stability when unfolded. Indeed, in the native status no aggregation was detectable (at least during 30 min) even at the highest *I*. This might be due either to the high positive charge of the proteins (see Table 1) or to their hydrophilicity. In addition, unfolding did not induce aggregation unless *I* was sufficient to screen the protein's charges. At 21 mM some of the charges were screened compared to the 2 mM samples. Nevertheless, no aggregation was observed at high temperature. Upon cooling, changes in the balance between electrostatic and polar interactions, likely associated with a

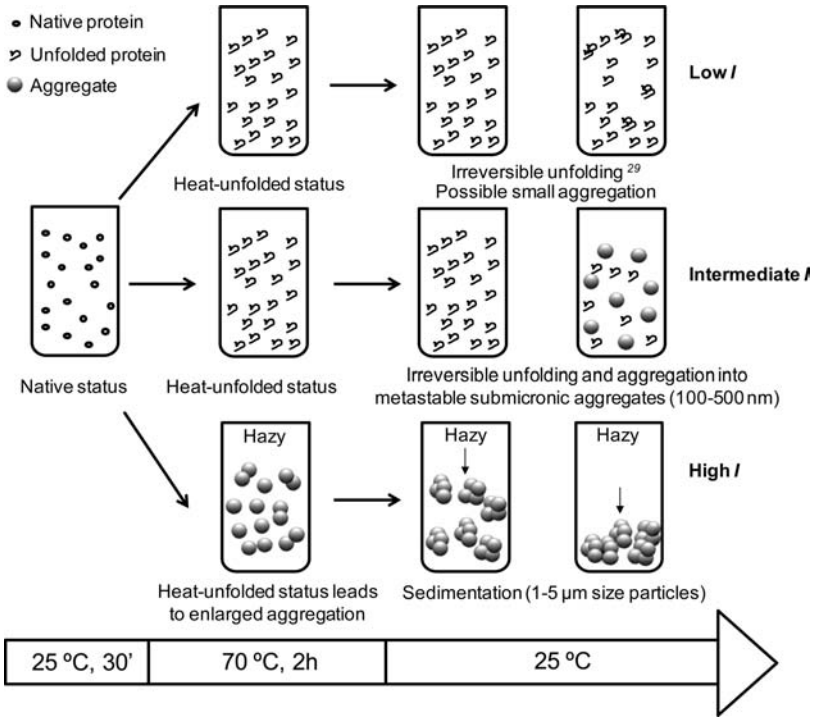


**Figure 3.** Schematic representation of the hypothetical effect of ionic strength and heating on the aggregation behavior of chitinases.

decrease of the thermal agitation, induced the onset of aggregation for M1. As stated before, aggregates formed very slowly and at a rate that progressively decreased as their size increased so that they remained submicronic for several days. There are two possible explanations for this behavior: (i) particle growth is related to attractive interactions between the first formed aggregates (primary aggregates), the aggregation rate of which is slower than the one of the single proteins due to their different structure (in terms of dimension and shape but also charge density or distribution as well as hydrophobicity/hydrophilicity), and smaller attractive interactions or (ii) aggregate growth is related to progressive deposition of proteins from the solution on the initially formed aggregates and thus its rate decreases with the "free" protein concentration.[30] Electrostatic repulsions between unfolded proteins were fully screened at 100 mM for M1, allowing protein aggregation to take place even at high temperatures. Aggregates were largely unstable, and (presumed) collisions led almost immediately to particle growth and to the formation of large micrometer-sized aggregates that quickly started to sediment (Figure 3, high *I*). Chitinase O behaved the same as chitinase M1, but higher ionic strengths were needed: 100 mM (instead of 21 for M1) to observe the formation of metastable aggregates upon cooling and 500 mM (instead of 100 for M1) to induce enlarged aggregation during heating.

On the basis of both their sequence and their fractionation behavior (Table 1 and Figure S1 of the Supporting Information), the differences observed between M1 and O were attributed to the fact that O is more charged than M1. As a matter of fact, M1 and O were separated by SCX (Supporting Information Figure S1A) because of this difference in charge, whereas, by HIC, their elution occurred at almost the same level of hydrophobicity (Supporting Information Figure S1,D).

**Impact of Ionic Strength on the Aggregation of Thaumatin-like Proteins.** The same experimental setup used for chitinases was adopted to study the effect of *I* on TL-proteins M2 and N. Proteins dissolved in model wine were heat tested at increasing *I*, and the haze formed and the protein content left in solution were measured (Figure 4). TL-proteins were less susceptible to *I* than chitinases. In fact, with *I* up to 100 mM TL-proteins did not form haze and the soluble protein content remained unaltered. *I* had an effect only on M2, but at a very high level (500 mM), at which about 90% of the protein precipitated (Figure 4B) and visible haze was formed (Figure 4A). DLS experiments performed on the same samples are shown in Figure 5.

M2 and N behaved similarly, at least in the normal wine ionic strength range.[19] With *I* up to 100 mM no significant changes in scattering intensity and particle sizes were observed for both proteins (Figure 5). A shift in $I/I_0$ was observed with *I* of 500 mM for protein N when the scattered intensity increased during heating from 1 to 3 and up to a plateau of 14 during cooling (Figure 5A). These results indicated that protein N underwent some changes upon heating and cooling. Some aggregates of about 400 nm formed during cooling (Figure 5B), but they were not measurable spectrophotometrically (Figure 4A) or visible to the naked eye. The protein content of N samples after analysis did not change (Figure 4B), supporting DLS findings and indicating that only a few aggregates had formed. Protein M2 was the only TL-protein giving haze at 500 mM *I* as soon as the heat was applied (Figure 5C,D).

M2 and N have the same melting temperature (61 °C, see Table 1) and, as stated for chitinases, the heat used here (2 h at 70 °C) should have been sufficient to unfold the proteins even at low *I*. Unlike chitinases, their unfolding was shown to be a reversible process.[29] As a consequence, after being unfolded during heating, upon cooling they return to a native or near-native status. If the *I* is high enough, aggregation can start during heating, preventing the reversibility of the unfolding (Figure 6). This may explain the behavior observed for protein M2 with 500 mM *I*. With protein N, 500 mM did not trigger large aggregation during heating, so that most of the proteins refolded

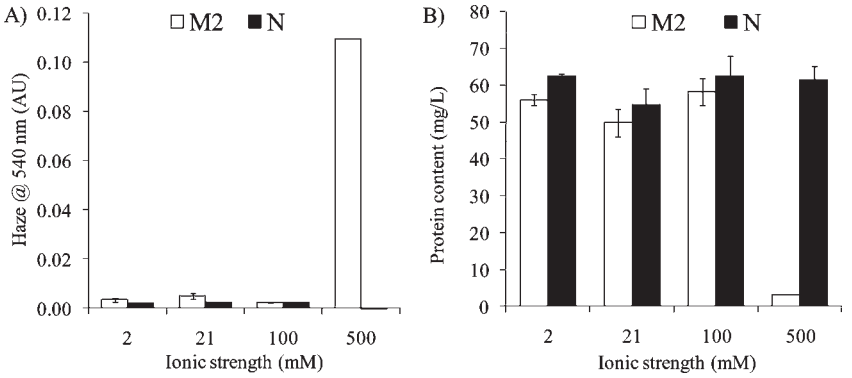


**Figure 4.** TL-proteins were dissolved in model wine containing increasing dosages of NaCl to obtain *I* levels of 2 mM (no salt), 21 mM (1.23 g/L NaCl), 100 mM (5.85 g/L NaCl), and 500 mM (29.25 g/L NaCl): (A) haze (at 540 nm) of samples after heat test (analyses were performed after samples were cooled for 20 h at 25 °C); (B) protein content (measured by EZQ) on supernatant obtained from centrifugation of samples (21000*g*, 15 min, 15 °C) after heat test. Protein contents in the untreated samples were 56.1 ± 2.9 and 62.5 ± 1.4 mg/L for M2 and N, respectively.

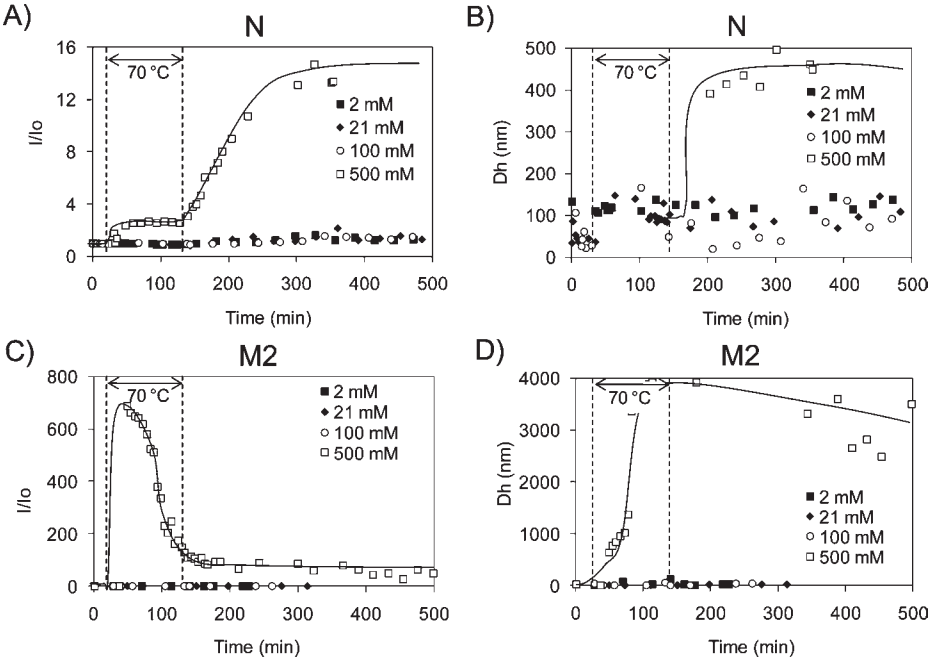


**Figure 5.** DLS measurement of proteins N and M2 with increasing *I*: (A) effect of ionic strength on normalized scattering intensity ($I/I_0$) for N; (B) particle size (Dh) of N at increasing *I*; (C) effect of ionic strength on $I/I_0$ for M2; (D) particle size (Dh) of M2 at increasing *I*. The protein content before the analysis was measured by the UV method and was between 93 and 104 mg/L for N and between 91 and 93 mg/L for M2. Lines represent the trends for samples at 500 mM *I*.

upon cooling. Therefore, only a few colloidal particles were formed and the sample remained limpid. As observed for chitinases, there is good agreement between the charge of TL-proteins and their sensitivity to ionic strength (Figure S1 of the Supporting Information and Table 1).

**Impact of Sulfate on the Aggregation of Purified Proteins.** Sulfate has recently been indicated as a possible required factor (factor X) for wine protein hazing.[9] The authors suggested that sulfate could play a role in protein denaturation, as opposed to protein aggregation, and demonstrated the involvement of sulfate in protein hazing for purified chitinases and TL-proteins in model wine (12% ethanol, 4 g/L tartaric acid, pH 3, *I* = 2 mM). Adding sulfate to this model wine triggered heat-induced protein aggregation, and the heat-induced haze increased with increasing sulfate concentrations. Because increasing levels of sulfate also resulted in an increased ionic strength, it needs to be elucidated whether protein aggregation was related to the increase in *I* or if sulfate has a different and specific effect. Therefore, we studied the role of increasing levels of sulfate (up to 4 g/L of $Na_2SO_4$, a value in excess of the normal content of sulfate in grape juices[31]) on the heat-induced protein aggregation/precipitation for each of the purified chitinase and TL-protein isoforms. Results were compared to those obtained at similar *I* via NaCl addition.

Data in Figure 7 show that both chitinase isoforms were very unstable in the presence of $Na_2SO_4$ at levels as low as 0.5 g/L (*I* = 10.5 mM). Even if the protein precipitation was almost complete for both proteins at 1 g/L of sulfate (Figure 7B), the level of haze kept increasing according to the dosage used (Figure 7A). These data are in agreement with results from a previous study in which sulfate was indicated as able to modulate protein hazing.[9] The

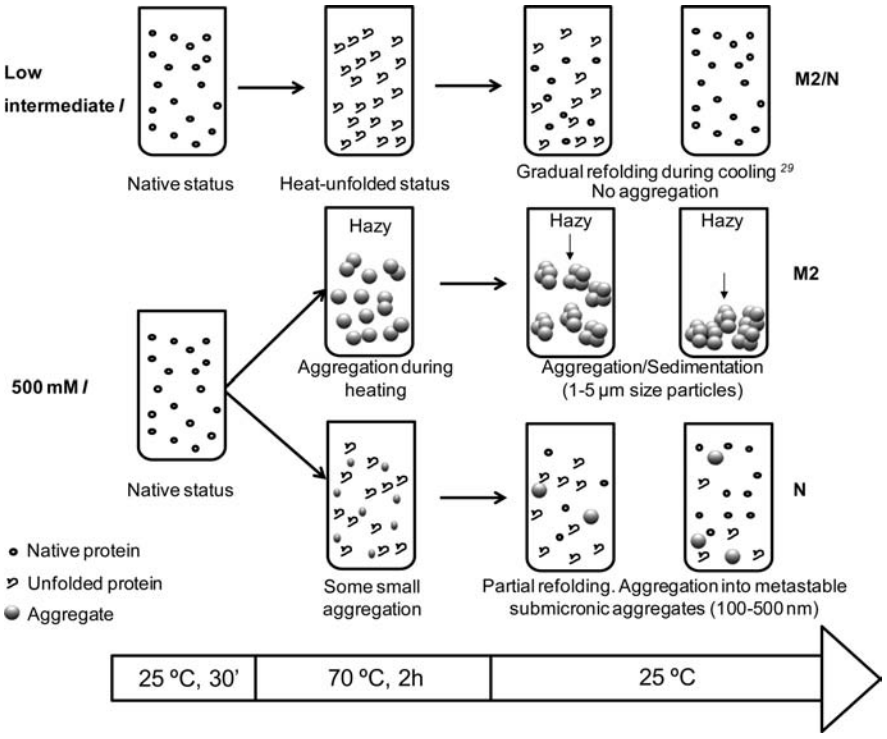


**Figure 6.** Schematic representation of the hypothetical effect of ionic strength levels and heating on the aggregation behavior of thaumatin-like proteins M2 and N.

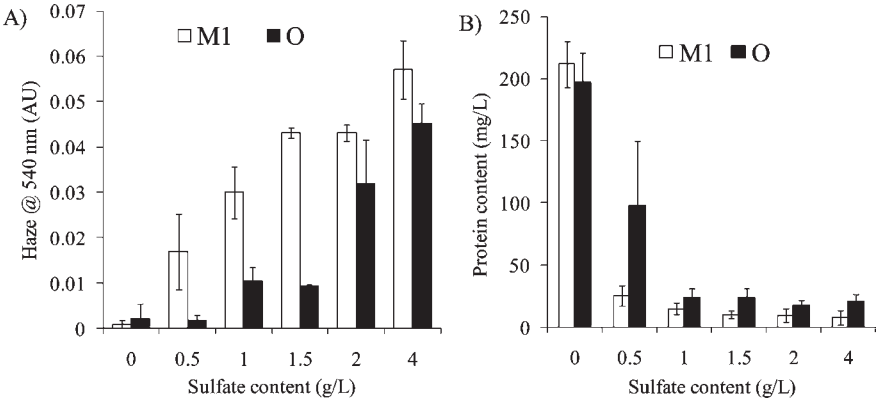


**Figure 7.** Chitinases were dissolved in model wine containing increasing dosages of $Na_2SO_4$ (from 0 to 4 g/L): (A) haze (at 540 nm) of samples after a heat test (analyses were performed after samples were cooled for 20 h at 25 °C; the ionic strength given by 1 g/L of $Na_2SO_4$ is 21 mM); (B) protein content (measured by EZQ) on supernatant obtained from centrifugation of samples (21000*g*, 15 min, 15 °C) after a heat test.

same experiments of Figure 7 were performed for TL-proteins M2 and N. In this case and contrarily to previous reports,[9] our results showed that increasing levels of $Na_2SO_4$ (up to 4 g/L, $I$ = 84 mM) had no effect on heat-induced aggregation of the two TL-protein isoforms: no variation in residual protein content or haze formation was observed (data not shown).

DLS experiments were performed for the four protein isoforms in the presence of 2 g/L $Na_2SO_4$ (Figure 8). DLS results were in agreement with those of Figure 7. With sulfate, M1 formed big visible aggregates as soon as the heat was applied. The ratio $I/I_0$ started increasing when the temperature reached 60−62 °C, up to values (1600) almost 10 times higher than those observed with NaCl at 500 mM $I$ (see Figure 2A). $I/I_0$ quickly reached a maximum before a drop caused by sedimentation. The trend of aggregation of chitinase O was different. $I/I_0$ increased upon heating to reach a plateau value of ≈200. Large micrometer-sized aggregates were formed; however, enlarged aggregation and sedimentation occurred only during cooling. An interesting observation was made with regard to the visual appearance of the aggregates from M1 and O (Figure 9), with O giving a homogeneous turbidity, whereas M1 gave a flocculated haze. For M1, additional experiments (not shown) performed with 2.5 g/L NaCl (same $I$ as 2 g/L $Na_2SO_4$) showed only limited aggregation, thus indicating that sulfate plays a different role that is independent of charge screening.

DLS experiments showed some limited aggregation for the two TL-protein isoforms during cooling (Figure 8C,D),

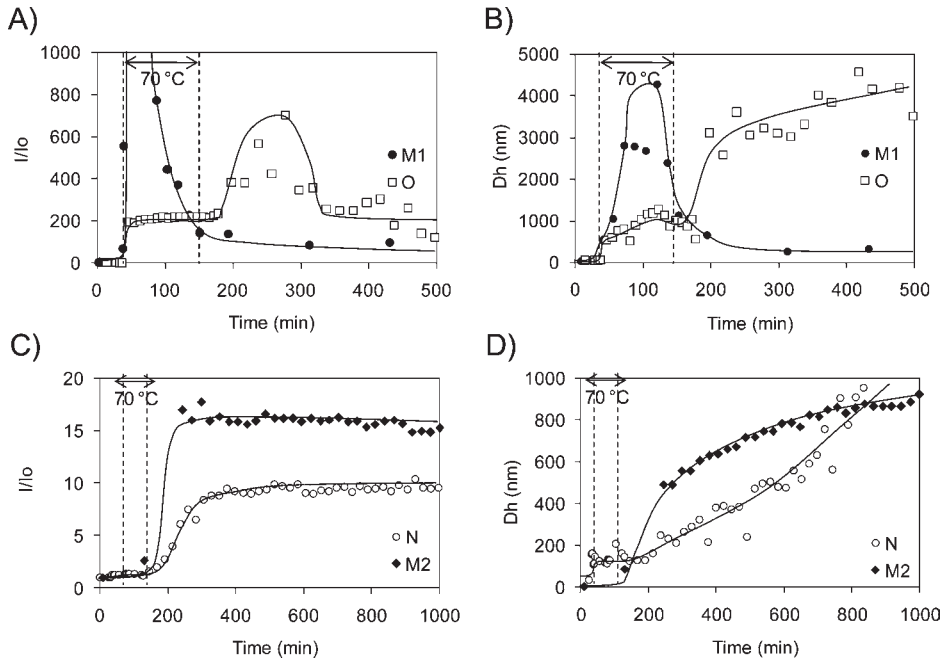


**Figure 8.** DLS measurements of proteins M1, O, N, and M2 in the presence of 2 g/L $Na_2SO_4$ ($I$ = 42 mM): (A) effect of sulfate on $I/I_0$ for M1 and O; (B) effect of sulfate on the particle size (Dh) of M1 and O; (C) effect of sulfate on $I/I_0$ for N and M2; (D) effect of sulfate on the particle size (Dh) of N and M2. The protein content before the analysis (measured by the UV method) was between 84 and 93 mg/L for M1, between 83 and 85 mg/L for O, between 104 and 114 mg/L for N, and between 91 and 93 mg/L for M2.

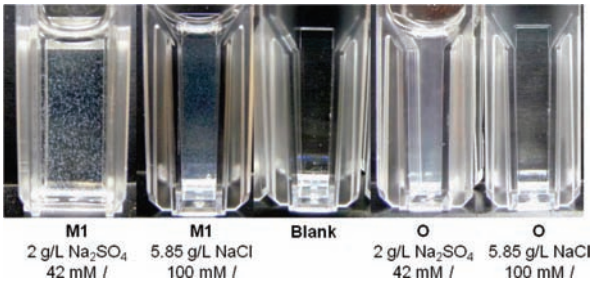


**Figure 9.** Visual assessment of samples after DLS analysis.

indicating a very different behavior between TL-proteins and chitinases toward this divalent ion. The scattering intensity quickly reached a plateau value, whereas the aggregate average size kept increasing progressively. Aggregate growth was thus related only to progressive collision and bridging of the aggregate formed upon cooling. Aggregates were large (≈1 $\mu$m) but still not visible, indicating that only a few were formed. This is in accordance with the analysis of the residual protein concentration of samples after heat tests when no changes could be detected (data not shown).

Sulfate is not needed for the unfolding of wine TL-proteins and chitinases upon heating.[29] Its role is thus to favor protein aggregation when unfolded, and its effect is much higher than the one obtained from monovalent ions at the same ionic strength that would provide similar screening of the repulsive electrostatic interactions.[20] In the Hofmeister series, sulfate is classified as a kosmotrope, as are all of the anions on the left of $Cl^-$. These kosmotropes are known to decrease protein (macromolecules) solubility in water (salting out effect). Although the exact molecular mechanisms involved in their effect are not fully understood yet, it has been proposed that it is the interaction of the anions with the hydration water that weakens the hydrogen bonding between water and macromolecules, so favoring salting out and thus aggregation.[32,33] However, such effects are usually observed for salt concentrations that are much higher than those used in the present work.[33] As a divalent ion, it can also be hypothesized that sulfate is able to cross-link different proteins and/or protein aggregates via ionic bonding.[18] This could explain its strong incidence on the rates of aggregation of chitinases as well as on the final size of the aggregates (Figures 9 and 10A). It should be pointed out that, with the same amount of protein aggregated, increasing sulfate concentration led to increasing turbidity, confirming its role in modulating the hazing of chitinases.

The role of sulfate can be schematized as shown in Figure 10. As observed for $I$, chitinases and TL-proteins behaved differently. During heating, sulfate was not able to efficiently bind the unfolded TL-protein isoforms, with only a few proteins affected. Upon cooling, most return to a native or near-native conformation, leaving only a few molecules "free" to be cross-linked by the sulfate to form aggregates (Figure 10B). Because this experiment was done in model wine, TL-proteins in their unfolded form could interact only with other protein molecules or with sulfate. Obviously, in real wine, TL-proteins would interact also with other solutes to form haze. This theory is supported by the detection of TL-proteins in hazes from real wines.[34]

By coupling heat test and DLS experiments we have obtained insights into the early stages of protein aggregation that are fundamental to fully understand the steps that lead to haze formation. It was demonstrated that the aggregation mechanisms of TL-proteins and chitinases are very different and strongly influenced by the ionic strength and the ionic content of the model wine.

It is a general belief that, in wines, protein unfolding leads to the exposure of their hydrophobic binding sites, and thus protein

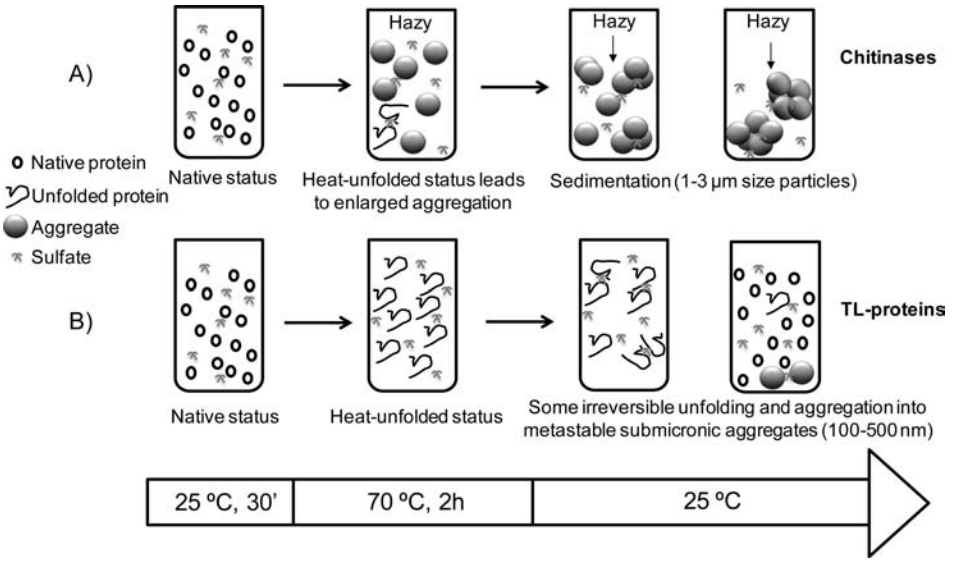


**Figure 10.** Schematic representation of the hypothetical effect of sulfate and heating on the aggregation behavior of chitinases (A) and thaumatin-like proteins (B).

aggregation is induced. However, it has been demonstrated that additional factors (factor X) are required for protein haze formation in wines.[9] Results obtained in our model systems indicate that the ionic strength is one of these factors. Indeed, the screening of electrostatic interactions was required to provoke significant heat-induced aggregation of the proteins and thus visible haze. The presence of sulfate greatly affected the colloidal status of proteins. Our data demonstrate that sulfate mediates the aggregation of unfolded chitinases, probably through a cross-linking action, during heating and/or cooling. As a matter of fact, sulfate induced aggregation upon heating and haze formation of chitinases at levels commonly found in wines (0.5 g/L of $Na_2SO_4$). On the contrary, TL-proteins did not form visible haze in the presence of sulfate but formed aggregates that in real wines could bind to other nonproteinaceous compounds to form haze. The sulfate−protein interaction could prevent the unfolding of TL-proteins from being reversible.

It is noteworthy that quite high ionic strengths (at least 100 mM but mostly 500 mM) were needed to induce the aggregation of some of the tested isoforms. Such ionic strengths are higher than that commonly observed in wines. In a similar experiment conducted in a real wine with ionic strength estimated to be ≈20 mM, the heat treatment led to visible haze.[13] However, in that wine, the role of the ionic strength in modulating the size of heat-induced aggregates was demonstrated. These observations indicate that ionic strength is likely of importance for the modulation of haze in real wines, even in the usual range found in wines. Moreover, all chitinases and most of the TL-proteins were heat-precipitated when the wine was submitted to a temperature of 70 °C, whereas this was not the case for TL-proteins in model systems. There are two possible explanations: (i) coprecipitation between the different protein classes takes place in real wines and (ii) other than proteins and salts, there are additional factors in wines that can trigger protein aggregation, as suggested in the literature.[8]

From a practical point of view, high salt concentrations in wines, possibly related to drought conditions, could favor an increase in protein hazing potential. If sufficiently high, this increase could also hinder the efficiency of bentonite fining by screening attractive electrostatic interactions between clay particles and wine proteins.

For a given protein class it seems possible to attribute differences in behavior to the protein isoform net charge. However, this does not account for the differences observed between TL-proteins and chitinases. Under our conditions, chitinases were more prone to precipitate and form haze than TL-proteins. This is in agreement with previous studies conducted in model solutions as well as in real wines.[13,29,35,36] From our data, chitinase appears to be the main contributor to haze formation in terms of absolute value. However, in this study TL-proteins and chitinases were analyzed at about the same concentration, even if in wines the concentration of chitinases is generally lower than that of TL-proteins.[37] Therefore, the real contribution of chitinases to the total haze could be lower than highlighted here.

Following the same approach adopted here, future studies will be focused on elucidating the role played by other wine compounds, such as polysaccharides and phenolic compounds, that are believed to participate in haze formation.

## ■ ASSOCIATED CONTENT

**Supporting Information.** Information about the protein purification method (Figure S1) and the protein identity purity as assessed by SDS-PAGE and RP-HPLC (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

*Phone: +33 (0)499612758. Fax: +33 (0)499612857. E-mail: vernhet@supagro.inra.fr.

## ■ ACKNOWLEDGMENT

Yoji Hayasaka, of the AWRI, is thanked for ESI-MS analysis. Marie Dufrechou and Stephanie Carrillo, INRA, are thanked for assistance in DLS experiments.

## ■ ABBREVIATIONS USED

SCX, strong cation exchange chromatography;; HIC, hydrophobic interaction chromatography; DLS, dynamic light scattering; $I$, ionic strength; $I/I_0$, ratio between scattering intensity/scattering intensity of the blank; Dh, mean hydrodynamic diameter; PI, polydispersity index; TL-proteins, thaumatin-like proteins; $T_m$, temperature of maximum apparent heat capacity.

## ■ REFERENCES


(1) Bayly, F. C.; Berg, H. W. Grape and wine proteins of white wine varietals. *Am. J. Enol. Vitic.* **1967**, *18*, 18–32.

(2) Hsu, J.-C.; Heatherbell, D. A.; Flores, J. H.; Watson, B. T. Heat-unstable proteins in grape juice and wine. II. Characterization and removal by ultrafiltration. *Am. J. Enol. Vitic.* **1987**, *38*, 17–22.

(3) Waters, E. J.; Wallace, W.; Williams, P. J. Heat haze characteristics of fractionated wine proteins. *Am. J. Enol. Vitic.* **1991**, *42*, 123–127.

(4) Waters, E. J.; Wallace, W.; Williams, P. J. Identification of heat-unstable wine proteins and their resistance to peptidases. *J. Agric. Food Chem.* **1992**, *40*, 1514–1519.

(5) Waters, E. J.; Shirley, N. J.; Williams, P. J. Nuisance proteins of wine are grape pathogenesis-related proteins. *J. Agric. Food Chem.* **1996**, *44*, 3–5.

(6) Waters, E. J.; Hayasaka, Y.; Tattersall, D. B.; Adams, K. S.; Williams, P. J. Sequence analysis of grape (*Vitis vinifera*) berry chitinases that cause haze formation in wines. *J. Agric. Food Chem.* **1998**, *46*, 4950–4957.

(7) Saywell, L. G. Clarification of wine. *Ind. Eng. Chem.* **1934**, *26*, 981–982.

(8) Waters, E. J.; Alexander, G.; Muhlack, R.; Pocock, K. F.; Colby, C.; O'Neill, B. K.; Høj, P. B.; Jones, P. Preventing protein haze in bottled white wine. *Aust. J. Grape Wine Res.* **2005**, *11*, 215–225.

(9) Pocock, K. F.; Alexander, G. M.; Hayasaka, Y.; Jones, P. R.; Waters, E. J. Sulfate − a candidate for the missing essential factor that is required for the formation of protein haze in white wine. *J. Agric. Food Chem.* **2007**, *55*, 1799–1807.

(10) Marangon, M.; Vincenzi, S.; Lucchetta, M.; Curioni, A. Heating and reduction affect the reaction with tannins of wine protein fractions differing in hydrophobicity. *Anal. Chim. Acta* **2010**, *660*, 110–118.

(11) Dupin, I. V. S.; McKinnon, B. M.; Ryan, C.; Boulay, M.; Markides, A. J.; Jones, G. P.; Williams, P. J.; Waters, E. J. *Saccharomyces cerevisiae* mannoproteins that protect wine from protein haze: their release during fermentation and lees contact and a proposal for their mechanism of action. *J. Agric. Food Chem.* **2000**, *48*, 3098–3105.

(12) Sarmento, M. R.; Oliveira, J. C.; Slatner, M.; Boulton, R. B. Influence of intrinsic factors on conventional wine protein stability tests. *Food Control* **2000**, *11*, 423–432.

(13) Dufrechou, M.; Sauvage, F.-X.; Bach, B.; Vernhet, A. Protein aggregation in white wines: influence of the temperature on aggregation kinetics and mechanisms. *J. Agric. Food Chem.* **2010**, *58*, 10209–10218.

(14) Record, M. T.; Zhang, W.; Anderson, C. F. Analysis of effects of salts and uncharged solutes on protein and nucleic acid equilibria and processes: a practical guide to recognizing and interpreting polyelectrolyte effects, Hofmeister effects, and osmotic effects of salts. *Adv. Protein Chem.* **1998**, *51*, 281–353.

(15) Von Hippel, P. H.; Wong, K. Y. Neutral salts. The generality of their effects on the stability of macromolecular conformations. *Science* **1964**, *145*, 577–580.

(16) Kohn, W. D.; Kay, C. M.; Hodges, R. S. Salt effects on protein stability: two-stranded alphahelical coiled-coils containing inter- or intrahelical ion pairs. *J. Mol. Biol.* **1997**, *267*, 1039–1052.

(17) Boye, J. I.; Alli, I.; Ismail, A. A.; Gibbs, B. F.; Konishi, Y. Factors affecting molecular characteristics of whey protein gelation. *Int. Dairy J.* **1995**, *5*, 337–353.

(18) Chi, E. Y.; Krishnan, S.; Randolph, T. W.; Carpenter, J. F. Physical stability of proteins in aqueous solutions: mechanisms and driving forces in non native protein aggregation. *Pharm. Res.* **2003**, *20*, 1325–1336.

(19) Cabanis, J. C.; Cabanis, M. T.; Cheynier, V.; Teissedre, J. L. Tables de compositions. In *OEnologie: Fondements Scientifiques et Technologiques*; Flanzy, C., Ed.; Lavoisier Tec & Doc: Paris, France, 1998; pp 318−326.

(20) Israelachvili, J. Electrostatic forces between surfaces in liquids. In *Intermolecolar and Surface Forces*, 2nd ed.; Israelachvili, J., Ed.; Academic Press: New York, 1991; pp 213−259.

(21) van Oss, C. J., Ed. *Interfacial Forces in Aqueous Media*; Dekker: New York, 1994.

(22) Bryant, C. M.; McClements, D. J. Influence of NaCl and $CaCl_2$ on cold-set gelation of heat-denatured whey protein. *J. Food Sci.* **2000**, *65*, 801–804.

(23) Pocock, K. F.; Rankine, B. C. Heat test for detecting protein instability in wine. *Aust. Wine Brew. Spirit Rev.* **1973**, *91*, 42–43.

(24) Van Sluyter, S. C.; Marangon, M.; Stranks, S. D.; Neilson, K. A.; Hayasaka, Y.; Haynes, P. A.; Menz, R. I.; Waters, E. J. Two-step purification of pathogenesis-related proteins from grape juice and crystallization of thaumatin-like proteins. *J. Agric. Food Chem.* **2009**, *57*, 11376–11382.

(25) Scopes, R K. Measurement of enzyme activity. In *Protein Purification Principles and Practice*, 2nd ed.; Springer-Verlag: Secaucus, NJ, 1987; pp 280−282.

(26) Marangon, M.; Van Sluyter, S. C.; Haynes, P. A.; Waters, E. J. Grape and wine proteins: their fractionation by hydrophobic interaction chromatography and identification by chromatographic and proteomic analysis. *J. Agric. Food Chem.* **2009**, *57*, 4415–4425.

(27) Hayasaka, Y.; Adams, K. S.; Pocock, K. F.; Baldock, G. A.; Waters, E. J.; Høj, P. B. Use of electrospray mass spectrometry for mass determination of grape (*Vitis vinifera*) juice pathogenesis-related proteins: a potential tool for varietal differentiation. *J. Agric. Food Chem.* **2001**, *49*, 1830–1839.

(28) Waters, E. J.; Wallace, W.; Williams, P. J. Identification of heat-unstable wine proteins and their resistance to peptidases. *J. Agric. Food Chem.* **1992**, *40*, 1514–1519.

(29) Falconer, R. J.; Marangon, M.; Van Sluyter, S. C.; Neilson, K. A.; Chan, C.; Waters, E. J. Thermal stability of thaumatin-like protein, chitinase and invertase isolated from Sauvignon blanc and Semillon juice, and their role in haze formation in wine. *J. Agric. Food Chem.* **2010**, *58*, 975–980.

(30) Stranks, S. D.; Ecroyd, H.; Van Sluyter, S.; Waters, E. J.; Carver, J. A.; von Smekal, L. Model for amorphous aggregation processes. *Phys. Rev. E* **2009**, *80*, art. no. 051907.

(31) Leske, P. A.; Sas, A. N.; Coulter, A. D.; Stockley, C. S.; Lee, T. H. The composition of Australian grape juice: chloride, sodium and sulfate ions. *Aust. J. Grape Wine Res.* **1997**, *3*, 26–30.

(32) Zhang, Y.; Cremer, P. S. Interactions between macromolecules and ions: the Hofmeister series. *Curr. Opin. Chem. Biol.* **2006**, *10*, 658–663.

(33) Cho, Y.; Zhang, Y.; Christensen, T.; Sagle, L. B.; Chilkoti, A.; Cremer, P. S. Effects of Hofmeister anions on the phase transition temperature of elastin-like polypeptides. *J. Phys. Chem. B* **2008**, *112*, 13765–13771.

(34) Esteruelas, M.; Poinsaut, P.; Sieczkowski, N.; Manteau, S.; Fort, M. F.; Canals, J. M.; Zamora, F. Characterization of natural haze protein in sauvignon white wine. *Food Chem.* **2009**, *113*, 28–35.

(35) Sauvage, F.-X.; Bach, B.; Moutounet, M.; Vernhet, A. Proteins in white wines: thermosensitivity and differential adsorbtion by bentonite. *Food Chem.* **2010**, *118*, 26–34.

(36) Marangon, M.; Van Sluyter, S. C.; Neilson, K. A.; Chan, C.; Haynes, P. A.; Waters, E. J.; Falconer, R. J. Roles of grape thaumatin-like protein and chitinase in white wine haze formation. *J. Agric. Food Chem.* **2011**, *59*, 733–740.

(37) Pocock, K. F.; Hayasaka, Y.; McCarthy, M. G.; Waters, E. J. Thaumatin-like proteins and chitinases, the haze-forming proteins of wine, accumulate during ripening of grape (*Vitis vinifera*) berries and

drought stress does not affect the final levels per berry at maturity. *J. Agric. Food Chem.* **2000**, *48*, 1637–1643.

## Notes

This work was supported financially by French−Australian Science and Technology Program (FAST) and by Australia's grapegrowers and winemakers through their investment body the Grape and Wine Research and Development Corporation, with matching funds from the Australian Government. The AWRI is a member of the Wine Innovation Cluster located in Adelaide.