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Roles of Proteins, Polysaccharides, and Phenolics in Haze Formation in White Wine via Reconstitution Experiments

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Supporting Information

ABSTRACT: Residual proteins in finished wines can aggregate to form haze. To obtain insights into the mechanism of protein haze formation, a reconstitution approach was used to study the heat-induced aggregation behavior of purified wine proteins. A chitinase, four thaumatin-like protein (TLP) isoforms, phenolics, and polysaccharides were isolated from a Chardonnay wine. The same wine was stripped of these compounds and used as a base to reconstitute each of the proteins alone or in combination with the isolated phenolics and/or polysaccharides. After a heating and cooling cycle (70 °C for 1 h and 25 °C for 15 h), the size and concentration of the aggregates formed were measured by scanning ion occlusion sensing (SIOS), a technique to detect and quantify nanoparticles. The chitinase was the protein most prone to aggregate and the one that formed the largest particles; phenolics and polysaccharides did not have a significant impact on its aggregation behavior. TLP isoforms varied in susceptibility to haze formation and in interactions with polysaccharides and phenolics. The work establishes SIOS as a useful method for studying wine haze.

KEYWORDS: *aggregation, chitinase, haze, phenolics, polysaccharides, protein stability, thaumatin-like protein, white wine*

INTRODUCTION

Protein haze formation in white wines is a serious quality defect because consumers perceive hazy wines as faulty. Protein haze is caused by the presence of residual grape pathogenesis-related (PR) proteins in wines after bottling, in particular, thaumatin-like proteins (TLPs) and chitinases.[1,2] It is widely accepted that protein haze formation in wine is associated with the elevated temperatures that the wines can be exposed to during storage or transportation, and this can affect the stability of the PR proteins, resulting in their aggregation into particles visible to the naked eye. Hence, PR proteins need to be removed from white wines, and this is performed through bentonite fining.

Research into alternatives to bentonite has been stimulated by the fact that this fining method has several drawbacks.[2] To find a valid substitute for bentonite, a better understanding of the mechanism of protein haze formation is required. It is currently proposed that protein instability in wines is a two-step phenomenon: protein unfolding, a temperature-mediated step, followed by colloidal aggregation, due to intermolecular interactions among unfolded proteins.[3] Understanding how differences in protein structure can affect protein stability is one of the keys to elucidating the physicochemical mechanisms involved in haze formation. In addition, nonprotein wine components have been shown to play a role in modulating protein hazing.[3−7] Despite recent advances in this field of research, several aspects of protein stability/instability in wines are still not fully understood. For instance, the influence of storage conditions and wine composition (i.e., temperature, pH, ethanol content, ionic strength, presence of cosolutes),[3,6−8] and the features of wine proteins (i.e., structure, molecular size, hydrophobicity) that are involved in their denaturation and interaction with other wine components, including polyphenols[9] and polysaccharides,[10,11] are not well understood.

The interaction of wine proteins with phenolic compounds has been the focus of extensive research for more than 50 years.[12] Phenolic compounds have been associated with wine protein haze as they interact with grape proteins[13,14] and as they have been found in heat-induced and natural hazes from various white wines.[9,15] Several studies suggest that hydrophobic bonding may be the major mode of interaction between condensed tannins and proteins[16,17] in particular, when the proteins are in unfolded status.[18] Some authors have reported that some polysaccharides[19−21] have a stabilizing effect toward heat-induced protein haze,[22] whereas others have found that polysaccharides could negatively affect wine stability.[8] However, the level of polysaccharides in these studies was much greater than that reported in wines.[23]

The study of the interactions between wine proteins and other wine molecules responsible for haze formation requires an accurate characterization of the size and concentration of the protein aggregates formed upon heating because both parameters determine the degree of wine turbidity. Protein aggregation has been mainly studied by nephelometry[24] and dynamic light scattering[3,6,7] techniques, whereas data on particle size were obtained using methods such as disk

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centrifugation,[25] gel electrophoresis,[26] or electron microscopy.[27] Flow cytometry[28] has a number of limitations, including the need for large sample volumes and the inability to accurately detect particles smaller than ~400 nm.[29] The concentration of nanoparticles is more difficult to measure,[30] but quantitative resistive pulse sensing using Coulter-type counters shows promise as a fast and accurate alternative to established sizing methods for nanoparticles.[27,31] A new instrument, the IZON qNano, utilizes scanning ion occlusion sensing (SIOS) to allow the detection of both the size and concentration of individual particles/aggregates. The qNano instrument incorporates (i) a tunable nanopore around which there is the membrane, a septum at the center of a cross-shaped stretching platform known as a "cruciform"; (ii) a fluid cell in which the cruciform is placed for ionic current measurements through the pore by using Ag/AgCl electrodes; and (iii) a U-tube manometer, which applies pressure across the membrane, enabling pressure-driven particle transport.[32] An obstruction in the nanopore creates a resistance in the electrical current, and this resistance is used to calculate the size, concentration, and mobility of the particles or molecules under investigation.

The aim of this study was to examine, via reconstitution experiments, the aggregation behavior of five purified wine proteins and to measure the size and concentration of individual particles formed by these proteins when heated in the presence or absence of wine phenolics and/or polysaccharides using SIOS.

MATERIALS AND METHODS

Materials. The wine used was produced in 2010 from a Chardonnay juice sourced from the Barossa Valley region (South Australia). No bentonite fining was performed.

Enological Analyses. Conventional enological analyses were performed by the Commercial Service of The Australian Wine Research Institute using standard methods (Table 1). Alcohol, pH, titratable acidity, and volatile acidity were measured using a Foss WineScan FT 120 as described by the manufacturer (Foss, Hillerød, Denmark). Free and total SO_2 were measured by the aspiration method.[33] Haze formed upon heat test was measured as indicated by Pocock and Waters.[34]

Table 1. Enological Parameters of the Chardonnay Wine

parameter	value
ethanol (% v/v)	12.3
pH	3.22
titratable acidity (g H_2SO_4/L)	6.0
total SO_2 (mg/L)	88
free SO_2 (mg/L)	8
volatile acidity (g/L)	0.22
sulfate (mg K_2SO_4/L)	310
haze upon heat test (NTU)	16.8

Purification of Wine Proteins. Chitinases and TLPs were isolated from 36 L of Chardonnay wine. Proteins were purified by strong cation exchange (SCX) followed by hydrophobic interaction chromatography (HIC) as described by Van Sluyter and colleagues.[35] SCX and HIC fractions (10 mL each) were pooled on the basis of elution profiles at A_{280} and reverse phase (RP) HPLC results. The purity and identity of collected fractions were assessed by RP-HPLC, SDS-PAGE, and peptide mass spectrometry (see the Supporting Information). Purified proteins, one *Vitis vinifera* class IV chitinase (named CHIT C), and four *V. vinifera* thaumatin like proteins (named TLP C, TLP D, TLP H, and TLP I) were stored as ammonium sulfate suspensions at 4 °C.

Purification of Polyphenols. Total polyphenols were captured by passing the unfined Chardonnay wine through a 4.6 × 50 cm FPX66 Amberlite column (Rohm and Haas Co., Philadelphia, PA, USA) and eluted with 80% (v/v) ethanol. The ethanol was evaporated under reduced pressure using a rotatory evaporator and the aqueous phenolic solution freeze-dried.

Purification of Polysaccharides. The protein-stripped wine (flow through from SCX step) was stripped of phenolics through two passages on a FPX66 column. The treated wine was concentrated 30-fold under reduced pressure, and total polysaccharides were precipitated by the addition of 3 volumes of cold ethanol. The pellet was collected by centrifugation (4000*g*, 15 min, 4 °C), dissolved in water, dialyzed (7 kDa MWCO) against water, and freeze-dried.

Preparation of the Base Wine. The flow through wine from the FPX66 Amberlite column was recovered, and macromolecules (>3 kDa) were removed by ultrafiltration with an Amicon Stirred Cell System (Millipore, Watford, UK).

Protein Preparation. Ammonium sulfate suspensions of purified proteins were centrifuged (13000*g*, 15 min, 4 °C), and the protein pellet was dissolved in ultrapure water. Salt removal and protein concentration were achieved by centrifugation with 3 kDa MWCO ultrafiltration Nanosep devices (Pall Corp., Glen Cove, NY, USA). Concentrated proteins were dissolved in the base wine and stored at 4 °C.

Protein Content Determination. Protein content was determined either by EZQ protein quantification kit (Invitrogen, Mount Waverley, VIC, Australia) as described previously[36] or by UV absorption at 260/280 nm.[37]

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE analyses were performed with NuPage 4–12% Bis-tris gels (Invitrogen, 1.5 mm thick, 15 wells) using an XCell SureLock Mini Cell (Invitrogen) as described previously.[6]

Reverse Phase (RP) HPLC. The purity of proteins was determined by RP-HPLC with a Vydac 250 mm × 2.1 mm i.d., 208TP52 RP-C8 column (Grace Davison Discovery Sciences, Baulkham Hills, NSW, Australia) on an Agilent 1200 system (Agilent Technologies, Santa Clara, CA, USA) according to the method of Van Sluyter et al.[35] Sample injection volumes were 25 μL. Protein identity was assigned by comparison of the retention times (RT) with those previously reported for purified grape PR proteins as follows: peaks with a RT between 12 and 16 min were assigned to the TLP class, whereas peaks eluted from 24 to 28 min were classified as chitinases.

Protein Identification by Peptide Mass Spectrometry. Bands from SDS-PAGE were excised and trypsin-digested according to the method of Shevchenko et al.[38] The resulting peptides were subjected to nanoLC-MS/MS and proteins identified with the GPM Manager and X!Tandem according to the method of Van Sluyter et al.[35] The protein database contained UniProt sequences downloaded September 10, 2011, from several sources: all reviewed *V. vinifera* sequences (151 sequences), sequences from the 12x V1 predictions of the VIGNE/VIGNA French/Italian grape genome project (29749 sequences), and the mitochondrial proteins from Picardi et al.[39] and Goremykin et al. (81 sequences).[40]

Analysis of Protein Aggregates with the IZON qNano. The instrument was used following the manufacturer's instructions. A detailed description of the technique is reported by Willmott et al.[32]

qNano and Membranes. Once the lower fluid cell was in place, 75 μL of base wine (used as the electrolyte) were placed into the middle channel. The upper fluid cell was set into place, and 40 μL of sample was added to it. A tunable nanopore targeting particles with a diameter range of 500–2000 nm was used. Voltage was adjusted until the current was approximately 140–150 nA, and samples were loaded.

Electrolyte and Standard Nanoparticles. Carboxylated polystyrene calibration standard particles with a diameter range of 500–2000 nm were diluted at the concentration of 5×10^7 particles/mL in base wine. The solution was sonicated at 20 °C in a sonicator bath for at least 5 min prior to use.

Data Conversion. Data were digitalized and interpreted using Izon's customized v. 2.2 instrument control software.

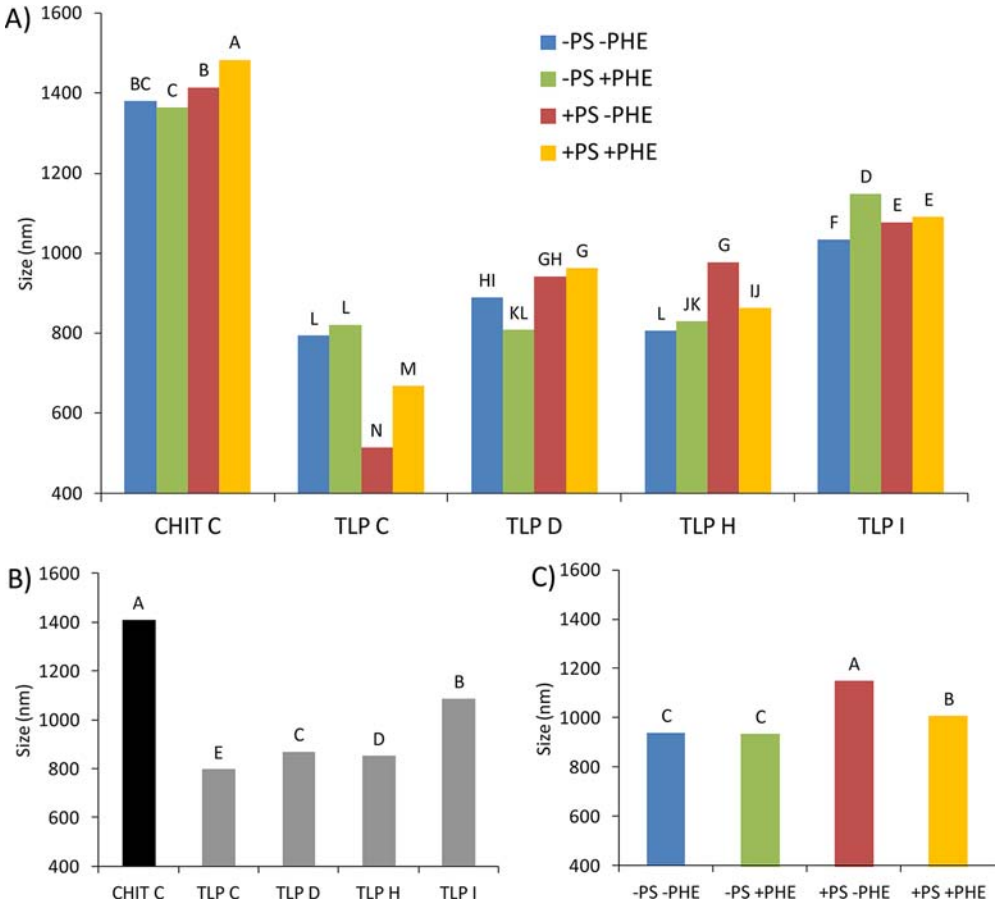

Figure 1. (A) Effect of protein and treatment (interaction protein × treatment) on the size (nm) of the aggregates formed upon heating/cooling cycles. (B) Effect of the proteins (CHIT C, TLP C, TLP D, TLP H, and TLP I) (main effect protein) on the size (nm) of the aggregates formed upon heating the samples. (C) Effect of the treatments (no addition of polysaccharides and phenolics, -PS -PHE; addition of phenolics, -PS +PHE; addition of polysaccharides, +PS -PHE; addition of polysaccharides and phenolics, +PS +PHE) (main effect treatment) on the particle size (nm) of the aggregates formed upon heating of the samples. The effects are significant at $P \leq 0.001$ according to ANOVA. Bars with different letters are significantly different according to the Tukey–Kramer HSD test ($P \leq 0.05$).

Experimental Design. Five proteins (CHIT C, TLP C, TLP D, TLP H, and TLP I), total polysaccharides (PS), and total phenolics (PHE), all purified from the same Chardonnay wine, were used in reconstitution experiments with base wine as the medium. Each protein was heat tested singularly or in combination with phenolics and/or polysaccharides for a total of four treatments for protein. Each compound was added to the base wine at the approximate concentration at which it was found in the original wine: proteins at 100 mg/L, polysaccharides at 170 mg/L, and phenolics at 225 mg/L. After the heating/cooling cycle (70 °C for 1 h and 25 °C for 15 h), the diameter (nm) and the concentration (particles/mL) of the aggregates formed in the different samples were determined by the qNano analysis. Control runs were done in the absence of proteins, and data from blank runs were not subtracted from the data of samples containing proteins. Base wine samples (without proteins/phenols/polysaccharides) did not form any particles.

Statistical Analysis. Each experiment was performed twice with independently prepared samples, whereas the analyses were done in triplicate (three independent measures for each combination of factor levels). Data collected from the qNano experiments were submitted to two-way analysis of variance (ANOVA) to examine the main effects "protein" and "treatment" and the effect of the interaction "protein × treatment" on the parameters considered (size and concentration of aggregates formed). Means were compared by the Tukey–Kramer HSD test ($P \leq 0.05$). Data were analyzed with CoHort software (CoStat, version 6.4).

RESULTS AND DISCUSSION

The qNano instrument was used to determine the aggregate size and concentration of the particles formed upon heating samples of base wine containing individual wine proteins, alone or in the presence of wine phenolics and/or polysaccharides.

Aggregate Size. Each time a particle passes through the nanopore, an electrical signal is registered by the system. The magnitude of the electrical blockade event (nA) is related to the particle volume, allowing the determination of the absolute size of the particles (nm) present in the sample.[32] In terms of particle size, several statistically significant differences were found: (i) among the treatments, in which each protein reacted differently (interaction effect protein × treatment) (Figure 1A); (ii) among the five proteins, regardless of the treatment to which they were subjected (main effect protein) (Figure 1B); and (iii) among the four treatments, regardless of single protein behavior (main effect treatment) (Figure 1C). All of the proteins were able to produce aggregates in the absence of the other components tested, indicating their ability to self-aggregate.

For all of the treatments considered, CHIT C was the protein producing the largest aggregates (average particle sizes about 1400 nm). The presence of phenolics or polysaccharides had no significant effects on the size of aggregates formed by

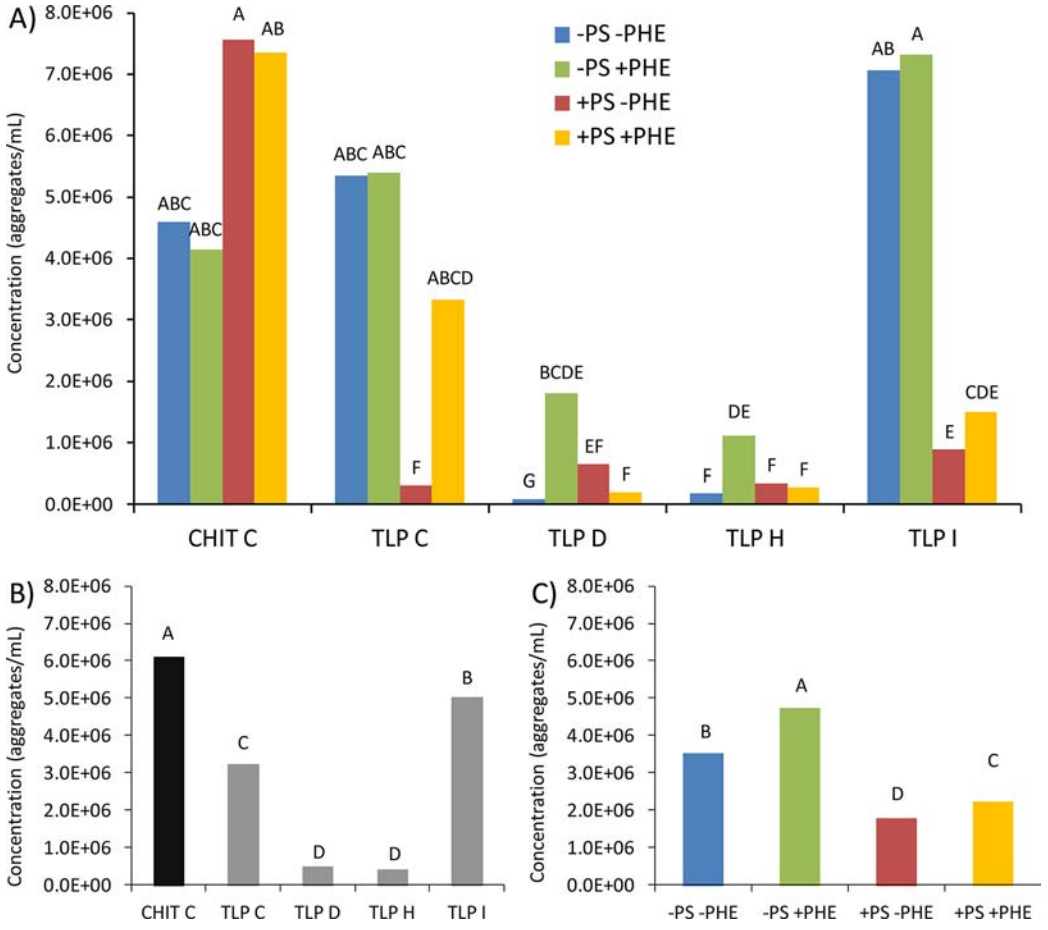

Figure 2. (A) Effect of protein and treatment (interaction protein × treatment) on the concentration (particles/mL) of the aggregates formed upon heating/cooling cycles. (B) Effect of the proteins (CHIT C, TLP C, TLP D, TLP H, and TLP I) (main effect protein) on the concentration (particles/mL) of the aggregates formed upon heating the samples. (C) Effect of the treatments (no addition of polysaccharides and phenolics, -PS -PHE; addition of phenolics, -PS +PHE; addition of polysaccharides, +PS -PHE; addition of polysaccharides and phenolics, +PS +PHE) (main effect treatment) on the concentration (particles/mL) of the aggregates formed upon heating of the samples. The effects are significant at $P \leq 0.001$ according to ANOVA. Bars with different letters are significantly different according to the Tukey–Kramer HSD test ($P \leq 0.05$).

CHIT C, whereas the combination of the two slightly increased aggregate size (Figure 1A).

Among the four TLP isoforms investigated, a great variability in aggregation behavior was observed.

TLP I was the isoform forming the largest particles, in particular, when phenolics were added. The presence of polysaccharides (either alone or together with phenolics) formed aggregates of intermediate size, whereas the smallest particles were observed when the protein was tested alone. TLP I differed statistically from the other three TLPs for each treatment.

Conversely to that observed for TLP I, TLP C (Figure 1A, blue bar) produced the largest aggregates when tested alone, and phenolics did not modify this behavior significantly (green bar). In contrast, the presence of polysaccharides alone or together with phenolics reduced significantly the size of the aggregates formed by this protein (red and yellow bars).

TLP D alone (blue bar) gave aggregates significantly larger than those formed in the presence of phenolics (green bar) but smaller than those detected when polysaccharides were added with phenolics to the base wine (yellow bars).

In contrast, TLP H alone (blue bar) produced particles that were significantly smaller than those produced after the addition of phenolics (green bar) and phenolics plus polysaccharides (yellow bar), whereas, in this case, polysaccharides alone caused the formation of the largest particles (red bar).

When the protein effect was considered (Figure 1B), CHIT C formed protein aggregates with mean diameters of approximately 1400 nm, significantly bigger than any of the four TLPs. Among these latter proteins, TLP I formed the largest aggregates (approximate 1100 nm mean diameter), whereas the other three TLPs (TLP C, TLP D, and TLP H) differed from each other but formed particles with a mean size between 800 and 900 nm.

In terms of treatment effect, the addition of phenolics alone did not modify the size of the particles formed (Figure 1C, compare green and blue bars). Conversely, the presence of polysaccharides with protein yielded the largest aggregates (average size of 1149 nm) (red bar), whereas the addition of both phenolics and polysaccharides to protein gave particles with an intermediate size (yellow bar).

When proteins were not added to the samples (control runs), the aggregation was very limited, with samples containing phenolics, polysaccharides, or both forming small particles with extrapolation of SIOS data denoting average sizes of 431, 482, and 474 nm, respectively, confirming that the

presence of proteins in the medium was required for the formation of large aggregates.

Concentration of Aggregates. The frequency of measured blockade events (counts/min) at the nanopore is directly proportional to the concentration of particles in the sample (particles/mL).[32] Several statistically significant differences in particle concentrations were found: (i) among the treatments, each protein reacted differently (interaction effect protein × treatment) (Figure 2A); (ii) among the five proteins, regardless of the presence of phenolics and/or polysaccharides (main effect protein) (Figure 2B); and (iii) among the four treatments (inclusion or not of phenolics and/or polysaccharides in the protein samples), regardless of the single protein behavior (main effect treatment) (Figure 2C).

CHIT C, TLP C, and TLP I self-aggregated, each forming >4.8 × 10^6 particles/mL. For CHIT C there was no significant effect on the number of aggregates formed when phenolics and/or polysaccharides were added (Figure 2A, compare green, red, and yellow bars with blue bars). Moreover, the addition of phenolics did not modify the number of aggregates formed by TLP C and TLP I when compared to the protein tested alone (compare green and blue bars), whereas the addition of polysaccharides significantly reduced the number of aggregated particles (compare red and blue bars). The combined presence of polysaccharides and phenolics resulted in a significant reduction in the number of particles when compared to the protein alone for TLP I, whereas this was not the case for TLP C (compare green and yellow bars).

The number of particles through self-aggregation of TLP D and TLP H was negligible (blue bars), whereas its maximum was observed in the presence of phenolics (green bars). The addition of polysaccharides significantly increased the amount of TLP D aggregates but not that of TLP H (compare red and blue bars), whereas in the presence of polysaccharides and phenolics the number of protein aggregates was very low (yellow bars).

Figure 2B shows the average number of aggregates formed by each protein (main effect protein) independent of the treatment applied. CHIT C was the protein that produced the highest number of aggregates upon heating, whereas, among TLPs, TLP I and TLP C produced significantly more aggregates than TLP D and H.

Looking only at the treatment effect, proteins alone produced an average of about 3.5 × 10^6 particles/mL (Figure 2C, blue bar). The presence of phenolics induced a significantly higher amount of aggregates (green bar), whereas the presence of polysaccharides resulted in a significant reduction of this quantity, especially when added without phenolics (red bar).

Control runs (done in the absence of proteins) always resulted in very low numbers of aggregates formed, with samples containing phenolics, polysaccharides, or both having average concentrations of 0.21 × 10^6, 0.20 × 10^6, and 0.42 × 10^6 particles/mL, respectively, indicating that the presence of proteins in the medium is crucial for the onset of aggregation.

Aggregates Total Volume. Because particle size and concentration were known, the total volume occupied by the aggregates for each sample tested could be calculated (Figure 3). Analysis of variance of the data showed that only the protein effect was significant, demonstrating that differences in total volumes of aggregates were not attributable to the treatment or to a combined effect between protein and treatment. In terms of total volume occupied by the aggregates, CHIT C aggregates were significantly larger than those of the four TLPs. Among

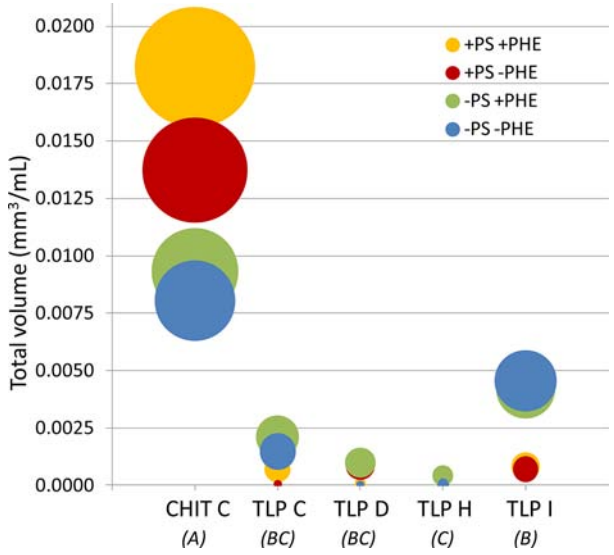

Figure 3. Calculation of the total volume occupied by the aggregates formed upon heating of samples containing CHIT C, TLP C, TLP D, TLP H, and TLP I and subjected to different treatments: no addition of polysaccharides and phenolics (-PS -PHE); addition of polysaccharides (+PS -PHE); addition of phenolics (-PS +PHE); addition of polysaccharides and phenolics (+PS +PHE). The main effect protein is significant at $P \leq 0.001$ according to ANOVA. Capital letters under the *x*-axis indicate the statistically significant differences ($P \leq 0.05$) among the total volume of aggregates occupied by proteins (independently by the treatment) according to the Tukey−Kramer HSD test.

TLPs, TLP I differed statistically from TLP H, whereas TLP C and TLP D were not different from any of the other TLPs.

Chitinase C. Independent of the treatment applied or the parameter measured, CHIT C always behaved differently from TLPs. In particular, it formed the largest size particles (Figure 1B) and the highest number of aggregates (Figure 2B). This chitinase was able to aggregate upon heating even in the absence of phenolics or polysaccharides. Indeed, when added alone in base wine (-PS -PHE treatment), CHIT C formed 4.5 × 10^6 of 1.4 μm diameter aggregates/mL, thus resulting in a very hazy sample. In previous experiments it was demonstrated that purified chitinases dissolved alone in model wine containing adequate ionic strength or sulfate could aggregate upon heating.[6,41] Protein−protein aggregation is generally described as a two-step process. First, the protein unfolds so that internal hydrophobic residues are exposed to the aqueous solvent. In the second step, the hydrophobic residues of the unfolded protein molecules interact to minimize the unfavorable exposure of the hydrophobic amino acid residues to the solvent.[42] In our case, protein unfolding was induced by heating, and protein aggregation is likely to have taken place via protein self-aggregation facilitated by the screening of protein charges by ions in base wine such as sulfate. Sulfate was present in the base wine at 310 mg/L and was previously shown to be required for haze formation in wine[4] and to be involved in the aggregation of heat-unfolded proteins.[6] It is likely that sulfate played a primary role in the extensive self-aggregation of CHIT C. Sulfate was also demonstrated to strongly affect chitinase aggregation in model wine upon heating, in which it modulated both the rate of aggregation and the size (1−5 μm) of the aggregates formed.[6,41] These observations are in agreement with results obtained here for CHIT C alone, which formed

aggregates ranging between 700 and 4050 nm. Because aggregates are visible to the naked eye only when they exceed 1000 nm in size,[1] it is further confirmed that chitinases play an active role in wine hazing.[6,41]

In general terms, it is likely that once CHIT C is in its irreversible heat-unfolded status, many hydrophobic binding sites remained exposed, so that nearly all of the unfolded protein molecules are involved in the self-aggregation, a process that is not hindered or favored by the presence of phenolics and/or polysaccharides.

TLP C and TLP I. In recent times TLPs and chitinases have been extensively studied in relation to their unfolding and aggregation behaviors. These studies indicate that the two classes have different unfolding temperature, refolding behavior,[43] and susceptibility to nonprotein factors present in the wine such as sulfate or salts.[6,41]

Two of the four TLP isoforms tested, TLP C and TLP I, produced aggregates more similar to those of CHIT C rather than those of the other two TLPs (Figure 2B). Both proteins formed a very high number of aggregates when heated alone in base wine (Figure 2A,B), indicating that self-aggregation of heat-unfolded proteins is significant for these two isoforms. The presence of phenolics did not significantly affect the number of aggregates formed (Figure 2A, compare green and blue bars). As phenolics bind proteins, some interaction would have been expected, but probably this could not be measured because the extent of self-aggregation for CHIT C, TLP C, and TLP I was high.

The presence of polysaccharides alone clearly inhibited the formation of aggregated particles (Figure 2A, compare red and blue bars). Indeed, polysaccharides have been reported previously to not contribute to haze formation upon heat test, and some polysaccharides classes (such as yeast mannoproteins) are considered to have a protective role toward wine protein hazing.[22] One hypothesis is that polysaccharides could interact with heat-unfolded proteins, thus preventing protein self-aggregation by limiting the availability of some protein binding sites with a steric hindrance mechanism.

The combined presence of polysaccharides and phenolics reduced the number of particles formed, in particular, for TLP I (Figure 2A, yellow bars). It is likely that polysaccharides and phenolics interact together, a fact resulting in the lower availability of polysaccharides to form complexes with proteins. Indeed, the ability of some polysaccharides to reduce the formation of protein−tannin aggregates is well documented.[11,44−46] Currently there are two proposed mechanisms to explain the inhibitory effect of polysaccharides toward protein−tannin aggregation: the molecular association in solution between polyphenols and polysaccharides, so that some of the polysaccharides are not available for inhibition of the protein aggregation; and the formation of a highly soluble ternary protein/polyphenol/carbohydrate complex resulting in a lower number of aggregates.[45] Our results indicate that both mechanisms could be valid for TLP I but not for TLP C, which had aggregate numbers that did not statistically decrease in the presence of polysaccharides (Figure 2A, compare yellow bar with green bar). Therefore, the inhibiting effect of polysaccharides toward protein−polyphenol interaction seems likely to be highly governed by the structure and the chemicophysical characteristics of individual wine proteins, with TLP isoforms behaving differently.

The size of the few TLP C aggregates formed in the presence of polysaccharides was significantly smaller than that of those formed in their absence (Figure 1A, compare red with blue and green bars). Furthermore, the presence of phenolics in combination with polysaccharides resulted in an intermediate size (Figure 1A, yellow bar), a phenomenon probably attributable to the fact that phenolics interacted with a portion of polysaccharides that was therefore not available to react with heat-unfolded proteins.

In terms of the size of aggregates, TLP I behaved differently from TLP C as it formed the largest aggregates in the presence of other molecules, especially when only phenolics were added (Figure 1A). However, for this protein, the presence of polysaccharides reduced the size of the polyphenols/protein aggregates, confirming the idea that the ternary complex protein/polyphenol/polysaccharide has a smaller size than that of the binary protein/polyphenol complex.[47]

It is of note to compare TLP C and TLP I: both formed a large number of aggregates (Figure 2A) but greatly differed in terms of aggregate size, with TLP C forming the smallest particles and TLP I the largest among TLPs (Figure 1B). These differences may be related to the great difference in surface charge shown by these two TLP isoforms, a feature that also affected their elution behavior during purification by cation exchange chromatography.

TLP D and TLP H. TLP D and TLP H behaved in a similar manner to each other but quite differently from the two TLPs discussed above.

Heating TLP D or TLP H alone in base wine yielded only a few aggregates, indicating that these proteins did not self-aggregate when in heat-unfolded status. It is possible that TLP D and TLP H have a low degree of unfolding/high degree of refolding, thus causing the exposure of only a few hydrophobic binding sites and resulting in little protein self-aggregation.

The presence of phenolics triggered the formation of a detectable number of aggregates for both proteins (Figure 2A), an occurrence that was not observed for the three proteins discussed above. As self-aggregation for TLP D and TLP H was very limited, they could interact with phenolics when in heat-unfolded status (Figure 2A, green bars). Compared to the proteins tested alone, the size of aggregates was lower in the presence of phenolics for TLP D, whereas for TLP H it was the opposite (Figure 1A). However, despite being significant, these differences were small in absolute value, indicating that phenolics have little effect in modulating the size of aggregates formed by these two TLPs.

Polysaccharides interacted with the heat-unfolded proteins but to a very low extent, with a statistically significant increase in number of aggregates only for TLP D (Figure 2A, compare blue and red bars) and a significant increase in size only for TLP H (Figure 1A, compare blue and red bars). The presence of phenolics and polysaccharides together yielded only a few aggregates (Figure 2A, yellow bars), suggesting that phenolics and polysaccharides interact with each other rather than with the proteins.

In general, the behavior of TLP D and TLP H toward heat unfolding seems similar to that of the TLPs described by Falconer et al.[43] It is likely that these proteins are not susceptible to self-aggregation and scarcely prone to bind to polysaccharides and phenolics because they have a higher unfolding temperature and/or higher rate of refolding upon cooling in comparison to TLP C and TLP I. Therefore, we can

speculate that TLP D and TLP H are not likely to be involved in wine protein hazing.

In conclusion, this study has demonstrated that SIOS, a new technology in the study of the protein aggregation, is a useful tool to understand the mechanism of aggregate formation in wine and yields valuable information on particle size and concentration. In particular, the coupling of this technique with the reconstitution approach allowed useful data to be obtained on the mechanism of protein aggregation under real wine conditions.

CHIT C was more easily unfolded by heat and, conversely to the TLPs tested, its substantial self-aggregation was not significantly affected by the presence of phenolics and/or polysaccharides, suggesting that, for chitinases, protein unfolding/aggregation is the main driver of hazing and that phenolics and polysaccharides have only a marginal role in this process.

The differences recorded in the aggregation behavior among isoforms of the same protein class (TLP) are noteworthy and demonstrated that it is possible to have large variability of behavior within this protein class. Differences among protein isoforms in terms of heat-induced unfolding, surface charge, and level of hydrophobicity should account for the differences in behavior among the isoforms of TLP. The most charged TLP, TLP I, as judged by cation exchange elution times, was the most prone to aggregate. TLPs as a group have been recently reported as having a less relevant role than chitinases in haze formation in wines,[41] but the data in the present study suggest that some TLP isoforms may participate more significantly than previously suspected.

In general, the role of chitinases as main contributors to protein aggregation was confirmed. Moreover, the observation that different isoforms of the same TLPs can have very different haze potentials could explain why certain wines are more susceptible to protein hazing than others. It is likely that, despite containing similar amounts of total TLPs, certain wines could contain a larger proportion of the more reactive TLP isoforms, thus resulting in greater instability. This fact might also explain why certain varieties (such as Sauvignon blanc) give white wines that are generally more unstable than others (such as Chardonnay) and why there are conflicting reports in the literature about the role of TLPs in haze formation. For TLPs in particular, nonprotein wine compounds (polyphenols and/or polysaccharides) were considered as having a crucial role in protein aggregation, leading to the final consideration that wine hazing is modulated by interactions between protein and other main wine molecules.[6,7] However, in this study we found that the type of protein was always more important than the effect of polyphenols and/or polysaccharides, and because our experiment simulated the real wine conditions, these findings are particularly relevant.

Our results confirmed the primary role of chitinases in wine hazing but also suggest a more important role for TLPs than previously thought. Having a better understanding of the role of all isoforms of wine proteins in haze formation will allow better predictive tools for haze potential of wines to be developed and improved and more targeted techniques to prevent haze formation in bottled white wines.

■ ASSOCIATED CONTENT

Supporting Information

Information about the protein purification method (Figure S1), the protein purity and identity as assessed by RP-HPLC (Figure S2), SDS-PAGE (Figure S3), and peptide mass spectrometry (Table S1), and the size measurements of aggregates are shown in Table S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

CHIT, chitinases; HIC, hydrophobic interaction chromatography; MWCO, molecular weight cutoff; NTU, nephelometric turbidity units; PHE, total wine phenolics; PR proteins, pathogenesis-related proteins; PS, total wine polysaccharides; SIOS, scanning ion occlusion sensing; TLP, thaumatin-like protein.

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