Roles of Grape Thaumatin-like Protein and Chitinase in White Wine Haze Formation

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Grape chitinase was found to be the primary cause of heat-induced haze formation in white wines. Chitinase was the dominant protein in a haze induced by treating Sauvignon blanc wine at 30 °C for 22 h. In artificial wines and real wines, chitinase concentration was directly correlated to the turbidity of heat-induced haze formation (50 °C for 3 h). Sulfate was confirmed to have a role in haze formation, likely by converting soluble aggregates into larger visible haze particles. Thaumatin-like protein was detected in the insoluble fraction by SDS-PAGE analysis but had no measurable impact on turbidity. Differential scanning calorimetry demonstrated that the complex mixture of molecules in wine plays a role in thermal instability of wine proteins and contributes additional complexity to the wine haze phenomenon.

KEYWORDS: Thaumatin-like protein; chitinases; haze; wine; grape juice

INTRODUCTION

White wine usually contains 10−500 mg/L protein (*1*−*3*). These proteins play an important role in the colloidal instability and clarity of white wines (*1*,*4*,*5*). The grape pathogenesis-related (PR) proteins, thaumatin-like proteins (TLP) and chitinases, are the major soluble proteins in grape juice (*6*) and have been deemed responsible for haze formation in wines (*4*, *7*−*9*). Due to their resistance to proteolysis, these proteins survive the fermentation process and remain in wine, where they can form insoluble aggregates resulting in the appearance of haze during storage (*10*−*12*). Although the mechanism for wine protein insolubility is not entirely understood, it is generally thought that the denaturation of wine proteins is due to unfavorable storage conditions. When the protein aggregates reach $\approx$1 μm diameter, they can be visually detected as a haze (*11*). Turbid wines do not present a health risk, but are visually unattractive and considered to be an indicator of poor quality by consumers.

Formation of wine protein haze has been studied by heat-inducing aggregation (*3*,*5*,*7*,*8*,*10*). Recent findings indicate that the temperature normally used to precipitate proteins ($\geq$80 °C) may be misleading because these temperatures may induce aggregation of wine proteins that are stable in wine at normal environmental temperatures (*13*). To our knowledge few studies have investigated the protein composition of hazes spontaneously formed during storage of wines (*14*−*16*). Esteruelas et al. showed

that a natural haze sourced from a Sauvignon blanc wine contained thaumatin-like protein, β-(1−3)-glucanase, and ripening-related protein grip22 precursor, although the results were inconclusive regarding the presence of chitinases (*16*).

It is widely thought that both the chitinases and TLPs are involved in wine haze formation (*11*, *12*). As the total protein content of a wine does not correlate with its heat instability, it is logical that the contributions of individual proteins to haze formation may vary. It has been demonstrated that a protein haze-forming potential can be modulated by several nonproteinaceous wine components such as metal ions, pH, ionic strength, polysaccharides, and phenolic compounds (*12*, *17*−*19*). Among the factors affecting protein stability, the sulfate anion has been proposed as an important factor (*17*), and its role in promoting protein aggregation has recently been confirmed (*19*). The modulating effect of the nonproteinaceous factors toward protein hazing justifies the contradictory results available in the literature regarding the relative importance of chitinases and TLPs in haze formation. Some studies indicate that the chitinases are the major haze-forming protein (*3*, *20*), whereas others argue that TLPs are responsible for wine hazes (*10*, *16*). Recent studies using purified grape proteins have allowed research into the mechanism of unfolding and aggregation of both TLPs and chitinases (*13*, *19*). It has been demonstrated that the two classes of proteins have different unfolding transition temperatures (T_m), different unfolding behaviors (irreversible for chitinases and reversible for TLPs), and different aggregation behaviors. The evidence supports the

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hypothesis that chitinases are more prone to form visible haze in model wine than thaumatin-like proteins (*13*, *19*).

The aim of the research presented in this paper was to further understand the roles that different proteins play during wine haze formation under conditions that a commercial wine could experience. The composition of the haze formed by exposing unfined wine to 30 °C for 22 h was carefully studied. Protein mixtures added to model and ultrafiltered real wines were then studied to better understand their behavior during heat treatment and to try to identify any interaction between the protein species. A theory that denatured proteins such as chitinase can coprecipitate other proteins was tested. Finally, differential scanning calorimetry (DSC) was used to verify whether the proteins behaved similarly in model and real wines.

MATERIALS AND METHODS

Materials. Two 2009 unfined wines (a Sauvignon blanc and a Chardonnay) from southeastern Australia were used for the haze composition and heat-induced haze formation experiments. The wines were donated by commercial producers, were made using standard winemaking practices on a commercial scale, except that bentonite fining to remove protein was not undertaken, and were stored below 10 °C before the experiments were undertaken. The model wine was prepared with 2 g/L malic acid, 12% (v/v) ethanol, pH 3.0. A 2009 commercial Sauvignon blanc wine from southeastern Australia was used for the DSC experiment.

Protein Purification. Chitinases and thaumatin-like proteins were isolated from Sauvignon blanc and Semillon grape juice sourced from South Australia. Proteins were purified by cation exchange and hydrophobic interaction chromatography, identified by peptide nanoLC-MS/MS, and identified as described by Van Sluyter et al. (*21*) (**Table 1**). Proteins were stored in ammonium sulfate suspension 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 deionized water. Salt removal and protein concentration were achieved via centrifugation with Nanosep 3 kDa ultrafiltration devices (Pall Corp., USA). Concentrated proteins were dissolved in model wine and stored at 4 °C.

Protein HPLC. Protein concentration and composition were determined by reverse-phase HPLC with a Vydac 2.1 × 250 mm C8 column (208TP52 Grace Davison Discovery Sciences, Australia) on an Agilent 1200 system according to the method of Marangon et al. (*22*) with modifications as suggested by Van Sluyter et al. (*21*). Injection volumes were 25 μL. From the 210 nm chromatogram, protein identity was assigned by comparison to retention times of purified grape PR proteins (*21*–*23*) as follows: peaks with a retention time between 12 and 16 min were assigned to the TL protein class, whereas peaks eluted from 24 to 28 min were classified as chitinases.

Protein Content Determination. Depending on the experimental conditions, four methods were used to measure the concentration of proteins: (i) when real or ultrafiltered wines spiked with proteins were examined, the KDS–BCA method was used (*24*); (ii) to determine protein concentrations after heat test of samples prepared in model wine, the BCA-200 protein assay kit (Pierce, Rockford, IL) was used according to the manufacturer's instructions; (iii) when proteins were pure, their content was determined by UV absorbance (*25*) at 280 nm (extinction coefficient calculated via http://ca.expasy.org/tools/protparam.html); (iv) RP-HPLC was used to measure the concentration of different classes of proteins by comparing their peak areas with that of a standard thaumatin (Sigma) (*22*).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was performed either with NuPage 4–12%, 10% or with 12% Bis-tris, 1.5 mm thick, 15-well gels (Invitrogen, Australia) 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. The pellet was separated from the supernatant via centrifugation (13000*g*, 15 min, 4 °C) for samples obtained after the heat test. The so obtained pellets were washed with model wine. Proteins from the supernatants were precipitated with the addition of 5 volumes of cold ethanol and centrifuged to obtain a pellet. In both cases the pellets were dissolved with NuPage loading buffer

Table 1. Characteristics of Purified Proteins

protein name[a]	protein type	T_m[b] (°C)
F1	class IV chitinase	55
F2	TL protein	56
I	VVTL1	62
D1 (O)	class IV chitinase	55

[a] Names from Van Sluyter et al. (*21*). [b] Data from Falconer et al. (*13*).

Table 2. Protein Content and Haze of the Unfined Sauvignon blanc Wine before (Control) and after Incubation at 30 °C for 22 h[a]

sample	protein content (mg/L)	haze (ΔNTU)
Sauvignon blanc control	289.5 ± 18.7	0.32 ± 0.04
Sauvignon blanc treated	252.7 ± 4.6	17.25 ± 0.56

[a] The protein content was measured after the haze was removed by centrifugation (15000*g*, 4 °C, 15 min), by the KDS−BCA method (BSA was used as the standard). Haze was measured by nephelometer. Each sample is the average of at least three replicates.

(Invitrogen NuPage recipe) containing 5% 2-mercaptoethanol. Precision Plus Protein unstained standards were from Bio-Rad. Proteins were stained with Pierce Imperial Protein Stain (Quantum Scientific, Australia) according to the manufacturer's microwave instructions.

Heat Test. Wines were heated at 50 °C for 3 h and cooled on ice for 2 h. After equilibration to ambient temperature, the haze was measured by calculating the difference between the heated and unheated samples in the absorbance values at 540 nm (*8*) by means of a spectrophotometer (Beckman DU 530 Life Science UV−vis spectrophotometer, Beckman Coulter, Fullerton, CA) or in NTU by means of a nephelometer (Hach model 2100N, Biolab Aust. Ltd., VIC, Australia) (*26*). Samples were considered to be protein unstable when the difference in absorbance between heated and unheated controls was > 0.02 absorbance unit (au) or 2 nephelometric turbidity units (NTU).

Peptide NanoLC-MS/MS and Database Searching. Bands from SDS-PAGE were excised and used for peptide nanoLC-MS/MS according to the method of Van Sluyter et al. (*21*). A ThermoFinnigan LTQ XL linear ion trap mass spectrometer was used. To create a wine protein database, all 76552 *Vitis* protein entries and 5693 EC1118 yeast proteins in NCBI were downloaded on February 12, 2010, and used with X!Tandem according to the procedure of Van Sluyter et al. (*21*).

Differential Scanning Calorimetry. Purified protein samples were adjusted to ~0.5 mg/mL with model wine or commercial wine and then dialyzed overnight at 4 °C with three changes of wine. DSC was undertaken with a VP-DSC (MicroCal, Northampton, MA) at a heating rate of 1.5 °C/min from 10 to 110 °C. The concentration of protein samples was between 0.02 and 0.04 mM. Samples and buffers were degassed by stirring under vacuum before loading into the DSC sample cell and reference cell. Data evaluation was done with the software provided by the manufacturer (Origin, version 7.0). Buffer−buffer baselines were subtracted from sample data.

RESULTS AND DISCUSSION

Protein Composition of Natural Wine Haze. Haze was induced by storing an unfined 2009 Sauvignon blanc wine at 30 °C for 22 h. This short incubation at a temperature well below the unfolding transition temperature (T_m) of the major PR-proteins (see **Table 1**) was sufficient to induce haze referred to in this document as a "natural" wine haze (**Table 2**). The decrease in soluble protein content of the treated wine was 13% (w/w). It is worth noting that 30 °C is a temperature markedly lower compared to the heat tests often used to assess wine stability, such as 80 °C for 6 h (*26*), still, short-term storage at 30 °C yielded a 17.3 NTU turbidity value, much higher than the threshold of 2 NTU accepted by the industry as denoting a hazy wine (*26*). This temperature is also likely to be experienced by wines during transport, especially when traveling in nonrefrigerated containers.
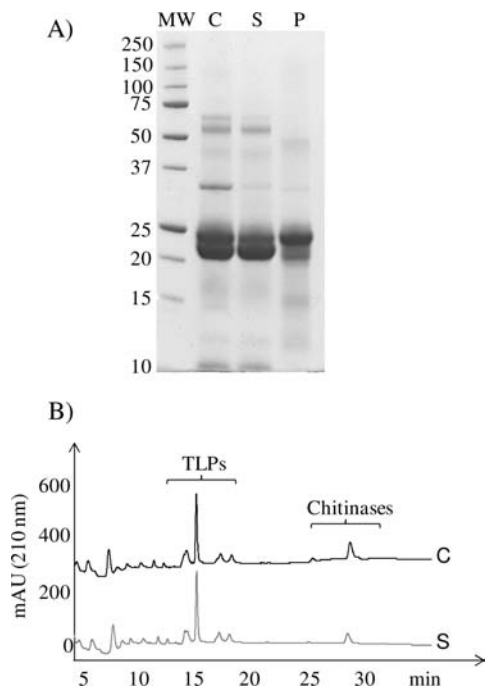
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Figure 1. Effect of incubation at 30 °C for 22 h on the protein composition of wine. (**A**) NuPAGE (12% Bis-tris) of proteins from Sauvignon blanc wine after 22 h at 30 °C. The wine was centrifuged (15000*g*, 15 min, 4 °C) and the obtained pellet washed with model wine. Proteins from 100 μL for control (before heating, lane C) and supernatant (after heating, lane S) and from 500 μL of pellet (after heating, lane P) were loaded per lane. (**B**) Reverse phase (C8) HPLC chromatograms of unheated Sauvignon blanc wine (C) and supernatant after 22 h at 30 °C (S). Proteins were reduced with 5% 2-mercaptoethanol.

The nature of the haze-forming proteins was investigated by SDS-PAGE (**Figures 1A** and **2**), HPLC (**Figure 1B**), and nanoLC-MS/MS (**Table 3** and Supporting Information Figure S1). Before heating, the wine was richer in TLPs than chitinases as shown by the higher intensity of the 22 kDa band in SDS-PAGE (**Figure 1A**, sample C) and the larger peaks in HPLC at a 15 min retention time (**Figure 1B**). The wine contained 59.3 mg/L TLPs and 33 mg/L chitinases (determined by HPLC). After incubation, the intensities of the SDS-PAGE bands with M_r of 35 and 25 kDa decreased (**Figure 1A**, sample S), and the missing proteins were found in the pellet (**Figure 1A**, sample P). HPLC analysis was not possible for the precipitated protein as the precipitate resolubilized poorly. The major band recovered in the haze was at 25 kDa, the correct position for chitinases, an assumption supported by the fact that the supernatant contained 24% less chitinases than the untreated control (as determined by HPLC peak area, **Figure 1B**). The level of TLPs remained almost unchanged, down by 3%. The gel also confirmed the heat stability of the band at around 60 kDa (assumed to be grape invertase) and the high heat sensitivity of the band at around 35 kDa, confirming the role of chitinase in haze formation seen in previous studies (*13*, *20*).

The identity of grape protein bands in SDS-PAGE gels has been previously established (*22*). Peptide nanoLC-MS/MS was used to establish the identity of the proteins in the natural wine haze. The insoluble haze fraction was washed, dissolved, and then loaded onto a SDS-PAGE gel (**Figure 2**) to obtain bands suitable for nanoLC-MS/MS analysis (**Table 3**).

The protein profile was the same as in **Figure 1A** even though this second separation was performed in nonreducing conditions. Proteins contained in the thickest bands (3−6, **Figure 2A**) were submitted to a second electrophoretic separation (**Figure 2B**). In
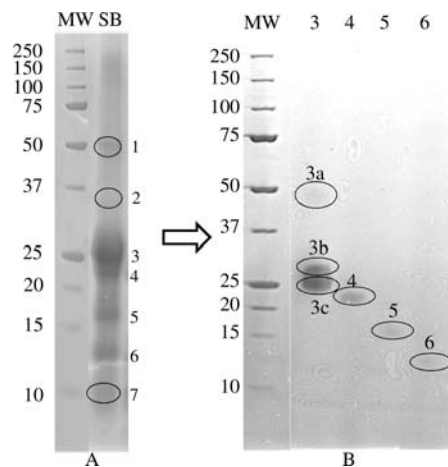

Figure 2. Separation of proteins in wine haze. (**A**) NuPAGE (10% Bis-tris) of the pellet formed after 22 h incubation at 30 °C of Sauvignon blanc (SB). Seven bands were excised. Faint bands (1, 2, and 7) were directly submitted to nanoLC-MS/MS analysis, whereas proteins of the thicker bands (3−6) were extracted by crushing the excised bands in the presence of 15 μL of NuPAGE loading buffer without 2-mercaptoethanol. After centrifugation (14000*g*, 15 min, 4 °C), the recovered proteins were directly reloaded onto a new gel (**B**). A total of nine bands (circled) were analyzed by nanoLC-MS/MS.

this way band 3 was separated into three further bands (named 3a, 3b, and 3c). This observation could indicate that the separation of proteins from a heterogeneous mix (i.e., the wine) can lead to misleading protein migration on PAGE or that the different bands in the second separation are artifacts of the extraction from the first gel. The reason for this occurrence could also be related to the reported different M_r of chitinases in nonreduced conditions (*10*, *27*). Whereas HPLC data (**Figure 1B**) showed that only the chitinase peak decreased in the supernatant, the presence of protein bands with SDS-PAGE mobility ranging from 52 to 9 kDa seemed to indicate that several classes of proteins were contained in the natural wine haze.

Results shown in **Table 3** indicate that almost every excised band, with the exception of bands 2 and 6, contained the same chitinase. Interestingly, no TLP was isolated from the natural wine haze. The band at 21 kDa (see band 4 in **Figure 2**), which would often be misidentified as TLP, was identified as a chitinase fragment. The nanoLC-MS/MS identification is in agreement with HPLC results (**Figure 1B**), which identified no significant loss of TLP from the wine. All of the bands with heterogeneous mobility were recognized as chitinases with the exceptions of band 2, which was identified as a *Saccharomyces cerevisiae* Bgl2p glucantransferase from the cell wall of yeast, and band 6, which was recognized as an exo-β-1,3-glucanase from *Vitis vinifera*. The presence of glucanases in wine hazes has recently been reported (*20*); to our knowledge this is the first report of a yeast protein contributing to a wine haze. The bands with diverse molecular weights identified as chitinase could be due to some protein degradation or the fact that the electrophoresis was performed under nonreducing conditions, which favor differences in the hydrodynamic volumes of the protein derived from structures stabilized by S−S bonds (*27*). The lack of resolution of the 9 kDa band (see Supporting Information Figure S1) indicates that this band could contain multiple proteolysis products of chitinase. The band at M_r of 50 kDa is likely a dimer of chitinase, as documented elsewhere (*10*, *22*).

These findings clearly indicate that in this wine and under these mild conditions the chitinases were the main proteins responsible

Table 3. Summary of the NanoLC-MS/MS Identification of Wine Protein Bands Excised from SDS-PAGE (See **Figure 2** and Supporting Information Figure S1)

band	M_r on SDS-PAGE[a] (kDa)	top ranked protein by X!Tandem[b]	unique/total peptides matched[c]	log(e)[d]
1	52	**gil33329392l class IV chitinase [*Vitis vinifera*]**	13/29	−196.5
		gil270235532l unnamed protein product [*Vitis vinifera*]	1/2	−20.2
		gil270254627l unnamed protein product [*Vitis vinifera*]	1/2	−11.8
2	35	**gil259146784l Bgl2p[*Saccharomyces cerevisiae* EC1118]**	6/20	−75
		gil225441373l PREDICTED: hypothetical protein [*Vitis vinifera*], glycosyl hydrolases family	3/8	−28
		gil33329392l class IV chitinase [*Vitis vinifera*]	3/5	−26.3
		gil270235532l unnamed protein product [*Vitis vinifera*]	2/4	−20.2
3a	50	**gil33329392l class IV chitinase [*Vitis vinifera*]**	6/9	−95.8
3b	29	**gil33329392l class IV chitinase [*Vitis vinifera*]**	21/46	−333.7
		gil270235532l unnamed protein product [*Vitis vinifera*]	1/2	−33.3
		gil259146784l Bgl2p [*Saccharomyces cerevisiae*]	1/2	−9.2
3c	25	**gil33329392l class IV chitinase[*Vitis vinifera*]**	28/60	−472.2
		gil2306813l class IV endochitinase [*Vitis vinifera*]	1/2	−424.7
		gil259146784l Bgl2p [*Saccharomyces cerevisiae*]	4/9	−45.9
		gil225441373l PREDICTED: hypothetical protein [*Vitis vinifera*], glycosyl hydrolases family	3/6	−44.4
4	21	**gil33329392l class IV chitinase [*Vitis vinifera*]**	17/35	−271.0
		gil225441373l PREDICTED: hypothetical protein [*Vitis vinifera*], glycosyl hydrolases family	7/14	−100.2
5	16	**gil33329392l class IV chitinase [*Vitis vinifera*]**	14/26	−214.2
		gil225441373l PREDICTED: hypothetical protein [*Vitis vinifera*], glycosyl hydrolases family	3/4	−31.6
6	13	**gil225441373l exo-β-1,3-glucanase [*Vitis vinifera*]**	3/3	−44.4
7	9	**gil33329392l class IV chitinase [*Vitis vinifera*]**	17/30	−259.7
		gil2306813l class IV endochitinase [*Vitis vinifera*]	1/1	−225.7
		gil225441373l PREDICTED: hypothetical protein [*Vitis vinifera*], glycosyl hydrolases family	6/11	−79.4
		gil270235532l unnamed protein product [*Vitis vinifera*]	4/5	−59.8
		gil259146784l Bgl2p [*Saccharomyces cerevisiae*]	4/6	−47.5

[a] Approximate M_r by SDS-PAGE under nonreducing conditions. [b] Protein identification number provided by the NCBInr database. [c] Total number of peptides identified by the X! Tandem program, which matched the identified protein. [d] Base −10 log of the expectation that the assignment is stochastic.

for haze formation. This observation is in agreement with some previous papers (*3, 13, 19, 20*). A possible explanation for this phenomenon can be found in differences between the half-life of denaturation of chitinases and TLPs. From the prediction made by Falconer et al. (*13*), the half-life of chitinases in model wine at 30 °C is 4.7 days and only 14 h at 35 °C, whereas the predicted half-life of TLPs is 45 years at 35 °C.

The natural wine haze used in this study was predominantly chitinase with a little Bgl2p and β-glucanase, which contradicts previously published work that claimed spontaneously formed wine hazes contain both TLPs and chitinases (*9, 16, 28*). There are two possible explanations for this apparent contradiction. It is possible that the thermally unstable chitinase is more prone to precipitate in the short term, whereas TLPs could precipitate in the long term with a slower and possibly different mechanism. The second hypothesis is that during their aggregation the chitinases could interact with the TLP and precipitate TLPs, which otherwise would remain soluble; in other words, coprecipitation may occur.

Purified Proteins in Model Wine. To undertake studies into chitinase and TLP behavior in a reproducible matrix, a model wine composed of 2 g/L malic acid and 12% (v/v) ethanol, pH 3.0, was used. The proteins were subjected to the modified heat treatment (50 °C for 3 h followed by cooling on ice for 2 h). Protein precipitation in wine has usually been studied by inducing haze at temperatures of 80 °C for ≥2 h (*8, 26*). At 80 °C, chitinases and TLPs experience temperatures that are about 20 °C above their T_m, conditions that are likely to cause the precipitation of classes of proteins (such as invertase) that would not participate to

the haze under normal conditions (*13, 20, 29*). The milder heating conditions used here are at the high end, but not outside, of the temperature range that bottled wines may encounter during transport and could be considered a more relevant heat challenge than 80 °C. The milder heating conditions also had the added benefit that they did not induce protein degradation due to acid hydrolysis that, at 80 °C, caused the appearance, by SDS-PAGE, of a ladder of low M_r degradation products (see Supporting Information Figure S2).

The coprecipitation theory (that denatured chitinase could coprecipitate TLPs that were otherwise soluble) was studied by adding purified TLP and chitinases to the model wine. Variable amounts of chitinase F1 (0, 10, 25, 40, and 50 mg/L) and a constant amount of TLP (50 mg/L) were added to each wine (**Figures 3** and **4**). The effect of sulfate addition was also studied by the addition of 0 or 2 g/L sodium sulfate. There was very little haze formation in the sulfate-free treatment groups. Haze correlated strongly with increasing chitinase in the presence of sulfate.

The most obvious observation is that sulfate was required for haze formation in model wines. Sulfate has been proposed as an essential factor (factor X) for haze formation in wine (*17*). The results in the current study are in agreement with previous papers in which sulfate at high concentrations was shown to have the ability to modulate the hazing of wine proteins (*17*).

The other obvious observation was that chitinase concentration in the presence of sulfate corresponded closely to haze formation as measured by turbidity at 540 nm (see **Table 4**). Indeed, there was a linear correlation ($R^2 = 0.99$) between chitinase content and

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haze formed when sulfate was added. The same samples were also analyzed by SDS-PAGE (**Figure 4**). In the absence of sulfate, the pellets always contained protein bands, indicating that some protein aggregation took place but that aggregates were not detected spectrally as haze (**Figure 3**) or visible to the naked eye. SDS-PAGE showed that when TLPs and chitinases were heated together, they both could be found in the precipitate captured in the pellet after centrifugation. The heat sensitivity of chitinase precipitation was confirmed by the presence of the chitinase band in all pellet fractions. Indeed, the only instance in which chitinases were found in the supernatant as well as the pellet fraction was when they were added alone to a model wine without sulfate (see Supporting Information Figure S3).

TLP does not play an obvious role in haze formation in the model wine (**Figure 3**), but interestingly there is a trace of TLP in the pellet when run on a SDS-PAGE gel (**Figure 4**). TLP I bands
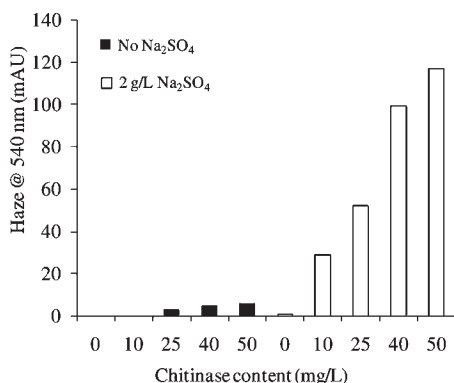

Figure 3. Effect of protein concentration and composition on haze formed in model wine. Haze formed after heat test (50 °C for 3 h) of model wine samples containing a fixed amount of TLP I (50 mg/L) and variable amount of chitinase F1 (0, 10, 25, 40, and 50 mg/L). Two series of samples were prepared with (black bars) or without (white bars) addition of 2 g/L Na₂SO₄.
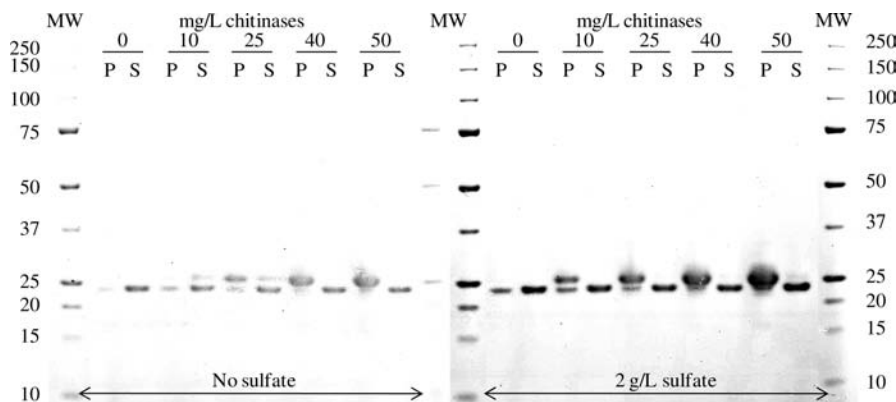
(M_r 22 kDa) were always found in the supernatants, in which they appeared as thick bands with constant intensity among samples. TLP I was consistently detected in the pellet but only as a faint band. On the contrary, chitinase F1 bands (M_r 25 kDa) were never detected in the supernatants in the presence of sulfate but only in pellets. It is known that upon heating chitinases unfold and are prone to aggregation (*13*). It is known that TLPs have, upon heating, a high degree of reversibility of unfolding, indicating that on cooling the protein returns to a native or near-native conformation (*13*). The small portion of proteins not returning to the native conformation could account for the small level of TLPs detected in the pellets.

Purified Proteins in Ultrafiltered Wine. To verify that the observations made in model wines are applicable to a white wine matrix, a protein-free wine matrix was prepared by removing its macromolecular fraction by ultrafiltration (MWCO < 3 kDa), leaving the low molecular weight component of the wine virtually intact.

The experiment used to study chitinase and TLP behavior in model wines was repeated using the ultrafiltered (UF) wine matrix. As in the model wine, there was an increase in haze formation in the sulfate treatment group related to chitinase concentration but, unlike the model wine, the treatment group without additional sulfate also had an increase in haze formation related to chitinase concentration (**Figure 5**). This is most likely due to endogenous sulfate in the UF wine. The average concentration is known to be around 0.3–0.4 g/L in Australian wines (*30*). The addition of 2 g/L sulfate resulted in greater haze formation; therefore, the sulfate in this wine is a limiting factor in the wine's haze-forming potential. The SDS-PAGE analyses of the supernatants and pellets after heat treatment of the chitinase- and TLP-spiked UF wine (**Figure 6**) are very similar to those in model wine (**Figure 4**). Again, the added sulfate played an important role in haze formation but had little impact on the protein composition of the pellet, as seen on the SDS-PAGE gel. Chitinase again plays the dominant role in haze formation, and

Figure 4. Soluble and insoluble protein composition, as assessed by NuPAGE (4–12% Bis-tris) after heating, of model wines containing purified proteins. After a heat test (50 °C for 3 h), each sample was centrifuged (15000*g*, 4 °C, 15 min) and the obtained pellet washed with model wine. Proteins from 100 μL for both supernatants (S) and pellets (P) were loaded per lane. Proteins were reduced with 5% 2-mercaptoethanol. Two series of wines were prepared with (gel on right) or without (gel on left) addition of 2 g/L Na₂SO₄.

Table 4. Summary of Sample Composition, Initial Protein Concentration, and Haze Formed after Heat Test in the Presence or Absence of Sulfate of Model Wine with Chitinase and TLP Added

ratio of chitinases/TLP	chitinases F1 (mg/L)	TLP I (mg/L)	total protein content (mg/L)	haze at 540 nm without sulfate (in mAU)	haze at 540 nm with sulfate (in mAU)
1:0	78	0	78	8	210
1:1.4	39	55	94	4	86
1:6	15.6	88	103.6	2	35
1:12	7.8	99	106.8	2	16
0:1	0	110	110	1	2

again the contribution of TLP is negligible. One observation for UF wine that is not mirrored in model wine is that the intensity of the TLP band in the pellet does seem to be linked to chitinase content (**Figure 6**). The observed TLP in the pellet may account for the reports of TLP being found in wine protein hazes, but TLP does not seem to play a role in the haze formation itself. Chitinase and sulfate are the dominant forces in haze formation. These data combined with those of previous works (*13*, *19*) indicate that the TLP and chitinase isoforms under investigation do not coprecipitate to a significant extent, at least when proteins are dissolved in a real wine matrix.

Matrix Effect on Grape Protein Stability. Results previously discussed highlighted the existence of some differences in the behavior of purified proteins that depend on the matrix in which they were dissolved. To obtain data regarding the possible effect of the wine matrix toward protein hazing, purified TLPs and chitinases were dissolved in UF wine and model wine and analyzed by DSC (**Table 5** and see Supporting Information Figure S4).

The DSC data indicated that all of the proteins tested had a lower unfolding temperature in real wine than in model wine, confirming the role of nonproteinaceous factors for protein stability (*17*, *18*). It has to be noted that the pH of the real wine was within 0.1 unit of the artificial wine and that the ethanol content was within 0.1%, minimizing the impact of differences in these variables. The unfolding transitions (T_m) of the two

chitinases tested (seen as a temporary rise in heat capacity as the protein unfolds) are at lower temperatures in real than in model wine, and in both cases proteins aggregate after unfolding (seen as a drop in heat capacity). Like chitinase, TLP unfolds at a lower temperature in real wine than in model wine, but it does not show a drop in heat capacity until around 100 °C (not shown), indicating that it can form aggregates under extreme conditions. This seems to be consistent and more pronounced in real wines than in artificial wines.

Respective Roles of Chitinase and TLP in Wine Haze Formation. Our results indicate that chitinases play the major role in haze formation in white wine at elevated temperatures. TLPs, on the other hand, never completely precipitated upon heating, and although these proteins could be found in the pellet after centrifugation, there was no strong evidence they contributed to the haze formation process. The linear relationship found between chitinase concentration and haze formation was demonstrated several times, and under various conditions (model and UF wines), with different protein types (F2 and D1, not shown) and concentration and with different ratios of TLPs and chitinases (see Supporting Information Figure S3). These findings lead to the hypothesis that chitinases are the most important proteins contributing to haze formation in unfined wines. However, it needs to be taken into account that TLPs are generally present in wines in higher concentration than chitinases. From the literature it is claimed that TLPs play a role in haze formation as well (*10*, *16*). It is possible that TLP isoforms from other varieties are structurally different from those investigated here, so that their contribution to haze is not simply temperature mediated. Other factors could also be involved in haze formation, such as metal ions and polyphenols. Whereas a role for TLP in wine haze formation cannot be totally excluded, the results presented here point to chitinase being the causative agent for heat-induced haze formation even at temperatures as low as 30 °C.
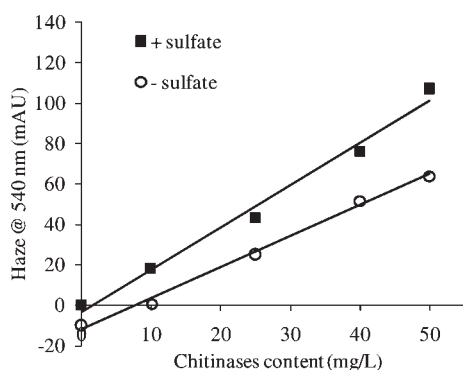

Figure 5. Correlation between net haze formed and initial chitinases content after heat test (50 °C for 3 h) of samples prepared in UF Chardonnay wine. Each sample contained a fixed amount of TLP I (50 mg/L) and a variable amount of chitinases F1 (0, 10, 25, 40, and 50 mg/L). Two series of samples were prepared with (■) or without (○) addition of 2 g/L Na$_2$SO$_4$.

Table 5. Melt Temperatures for Unfolding of TLPs F2 and I and the Chitinases F1 and O (for Details of the Curves See Supporting Information Figure S4)

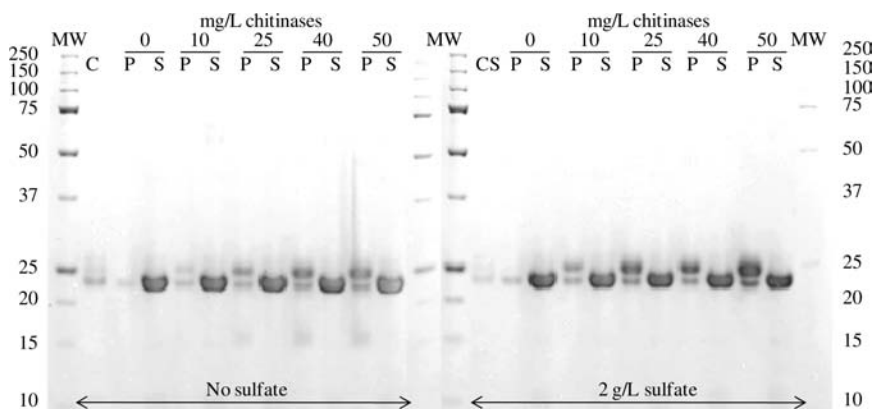|	T_m (°C)			
	chitinase F1	chitinase O[a]	TLP F2	TLP I
model wine	53.3	48.1	52.8	60.6
real (UF) wine	51.3	45.6	51.8	58.2

[a] Chitinase O is the same as D1 (*13*, *19*).

Figure 6. Soluble and insoluble protein composition, as assessed by NuPAGE (10% Bis-tris) after heating, of an ultrafiltered Chardonnay wine containing added purified proteins. Each sample was centrifuged (15000*g*, 4 °C, 15 min) and the obtained pellet washed with model wine. Proteins from 100 μL for control (C, ultrafiltered wine alone; CS, ultrafiltered wine with sulfate), supernatants (S), and pellets (P) were loaded per lane. Proteins were reduced with 5% 2-mercaptoethanol. Two series of wines were prepared with (gel on right) or without (gel on left) addition of 2 g/L Na$_2$SO$_4$.

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In the literature it is suggested that the mechanism of action of sulfate in protein haze formation is due to its ability to cross-link heat unfolded proteins to promote their aggregation or precipitation (*17*, *19*). The results shown here suggest that without sulfate present, TLP and chitinase will form soluble aggregates too small to scatter light and cause turbidity.

One of the main theories tested was the fact that TLPs and chitinases, being usually found in hazes together, could have precipitation behaviors that are somehow linked. From our experiments the coprecipitation theory has not been disproved for real wines but has been shown to be insignificant. The differences in T_m observed in different media confirm that the stability of chitinases and TLPs is affected by the matrix, a fact that could account for the differences in hazing behavior reported in the literature among different wines.

The differences in T_m observed between model and real wine confirms that the stability of chitinases and TLPs is greatly affected by the nonproteinaceous compounds of the matrix. Several authors have already made this assumption (*17*, *18*), but DSC results shown here are a clear demonstration of this. In addition, our findings contribute to the explanation of why the haze behavior of wine is reported to be so heterogeneous (*11*, *12*). Chitinases and TLPs are constitutively expressed during grape ripening and therefore recoverable from nearly all grape juice (*31*, *32*). However, despite containing the same classes of proteins, certain wines (i.e., Sauvignon blanc, Semillon) are more likely to be unstable than others (i.e., Riesling, Prosecco). By considering a wine as a system constituted of haze-forming proteins and nonproteinaceous factors (matrix), it is possible that the proteins are the less variable factor among wines, whereas it is the matrix that varies the most. This variation, as demonstrated by DSC data, could account for the differences in hazing behavior of similar proteins in different wines.

ABBREVIATIONS USED

PR-proteins, pathogenesis-related proteins; TLP, thaumatin-like protein; VvTL, *Vitis vinifera* thaumatin-like protein; BSA, bovine serum albumin; NTU, nephelometric turbidity unit; MWCO, molecular weight cutoff; M_r, relative mobility; UF, wine, ultrafiltered wine; DSC, differential scanning calorimetry; HPLC, high-performance liquid chromatography; T_m, temperature of maximum apparent heat capacity.

Supporting Information Available: Sequence coverage of nanoLC-MS/MS data (Figure S1), SDS-PAGE of purified proteins heat tested for different lengths (Figure S2), SDS-PAGE of purified proteins after heat test in model wine (Figure S3), and details of DSC scans (Figure S4). This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

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

(2) Santoro, M. Fractionation and characterization of must and wine proteins. *Am. J. Enol. Vitic.* **1994**, *46*, 250−254.

(3) Vincenzi, S.; Polesani, M.; Curioni, A. Removal of specific protein components by chitin enhances protein stability in a white wine. *Am. J. Enol. Vitic.* **2005**, *56*, 246−254.

(4) Hsu, J. C.; Heatherbell, D. A. Isolation and characterization of soluble proteins in grapes, grape juice and wine. *Am. J. Enol. Vitic.* **1987**, *38*, 6−10.

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

(6) Tattersall, D. B.; Van Heeswijck, R.; Hoj, P. B. Identification and characterization of a fruit-specific, thaumatin-like protein that accumulates at very high levels in conjunction with the onset of sugar accumulation and berry softening in grapes. *Plant Physiol.* **1997**, *114*, 759−769.

(7) Dawes, H.; Boyes, S.; Keene, J.; Heatherbell, D. Protein instability of wines: influence of protein isoelectric point. *Am. J. Enol. Vitic.* **1994**, *45*, 319−326.

(8) 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.

(9) 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.

(10) Vincenzi, S.; Marangon, M.; Tolin, S.; Curioni, S. Protein evolution in a white wine during the early stages of winemaking and its relations with wine stability. *Aust. J. Grape Wine Res.* **2010**, DOI: 10.1111/j.1755-0238.2010.00113.x

(11) Ferreira, R. B.; Piçarra-Pereira, M. A.; Monteiro, S.; Loureiro, V. B.; Teixeira, A. R. The wine proteins. *Trends Food Sci. Technol.* **2002**, *12*, 230−239.

(12) 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.

(13) 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.

(14) Koch, J.; Sajak, E A Review and some studies on grape protein. *Am. J. Enol. Vitic.* **1959**, *10*, 114−123.

(15) 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.

(16) 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.

(17) 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.

(18) Batista, L.; Monteiro, S.; Loureiro, V. B.; Teixeira, A. R.; Ferreira, R. B. The complexity of protein haze formation in wines. *Food Chem.* **2009**, *112*, 169−177.

(19) Marangon, M.; Sauvage, F.-X.; Waters, E. J.; Vernhet, A. Effects of ionic strength and sulfate upon thermal aggregation of grape chitinases and thaumatin-like proteins in model system. 2011, in preparation.

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

(21) 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.

(22) 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.

(23) Peng, Z.; Pocock, K. F.; Waters, E. J.; Francis, I. L.; Williams, P. J. Taste properties of grape (*Vitis vinifera*) pathogenesis-related proteins isolated from wine. *J. Agric. Food Chem.* **1997**, *45*, 4639−4643.

(24) Vincenzi, S.; Mosconi, S.; Zoccatelli, G.; Dalla Pellegrina, C.; Veneri, G.; Chignola, R.; Peruffo, A.; Curioni, A.; Rizzi, C. Development of a new procedure for protein recovery and quantification in wine. *Am. J. Enol. Vitic.* **2005**, *56*, 182−187.

(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) Pocock, K. F.; Waters, E. J. Protein haze in bottled white wines: how well do stability tests and bentonite fining trials predict haze formation during storage and transport? *Aust. J. Grape Wine Res.* **2006**, *12*, 212−220.

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Marangon et al.

(27) Vincenzi, S.; Curioni, A. Anomalous electrophoretic behaviour of a chitinase isoform from grape berries and wine in glycol chitin-containing SDS-PAGE gels. *Electrophoresis* **2005**, *26*, 60−63.

(28) 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.

(29) 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.

(30) 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.

(31) Derckel, J. P.; Legendre, L.; Audran, J. C.; Haye, B.; Lambert, B. Chitinases of the grapevine (*Vitis vinifera* L.): five isoforms induced in leaves by salicylic acid are constitutively expressed in other tissues. *Plant Sci.* **1996**, *119*, 31−37.

(32) Robinson, S. P.; Jacobs, A. K.; Dry, I. B. A class IV chitinases is highly expressed in grape berries during ripening. *Plant Physiol.* **1997**, *114*, 771−778.