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Thermal Stability of Thaumatin-Like Protein, Chitinase, and Invertase Isolated from Sauvignon blanc and Semillon Juice and Their Role in Haze Formation in Wine

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A thermal unfolding study of thaumatin-like protein, chitinase, and invertase isolated from *Vitis vinifera* Sauvignon blanc and Semillon juice was undertaken. Differential scanning calorimetry demonstrated that chitinase was a major player in heat-induced haze in unfined wines as it had a low melt temperature, and aggregation was observed. The kinetics of chitinase F1 (Sauvignon blanc) unfolding was studied using circular dichroism spectrometry. Chitinase unfolding conforms to Arrhenius behavior having an activation energy of 320 kJ/mol. This enabled a predictive model for protein stability to be generated, predicting a half-life of 9 years at 15 °C, 4.7 days at 30 °C, and 17 min at 45 °C. Circular dichroism studies indicate that chitinase unfolding follows three steps: an initial irreversible step from the native to an unfolded conformation, a reversible step between a collapsed and an unfolded non-native conformation, followed by irreversible aggregation associated with visible haze formation.

KEYWORDS: Grape; denaturation; aggregation; unfolding; calorimetry; instability

INTRODUCTION

Clouding, haze-formation, and precipitation are aesthetic problems in white wines which are caused by the presence of grape proteins (*1*). Since the 1930s, fining with bentonite clay has been used to clarify and stabilize white wines (*2*) through the removal of problematic grape proteins (*3*). Experience has led to the understanding that haze formation in unfined wine is generally associated with the elevated temperatures that can occur during storage or transportation. The heat test commonly used for wine stability uses elevated temperatures for several hours to induce a visible haze as the basis for assessing wine stability (*4*), and fined wines are often referred to as being heat-stable. The early work on identifying the proteins responsible for haze formation used heat-induced aggregation to isolate the "haze-forming" proteins from unfined wines (*5, 6*). The heat-induced haze-forming proteins have been identified as chitinases and thaumatin-like proteins (TLP) (*7, 8*). These pathogenesis-related proteins are likely present in grapes to counter fungal infection; they survive the winemaking process and remain in finished wine if the bentonite fining process is not used. Analysis of naturally occurring deposits in a Sauvignon blanc wine has confirmed the presence of thaumatin-like protein, β-(1−3)-glucanase, and ripening-related protein grip22 precursor but was inconclusive regarding the presence of any of the chitinases

in the aggregate (*9*). The naturally occurring deposits also contained phenolic compounds and polysaccharides indicating that the haze-forming proteins interact with other molecules in the wine (*9*), which may affect protein stability and aggregation. Phenolic compounds have been associated with natural and heat-induced protein haze in other wines (*10*). Multifactorial experimentation has also determined that sulfate cations can play a role in haze formation (*11*). It would be fair to conclude that protein instability in wine is the result of a complex interplay between a range of proteins and chemical and physical factors.

The problem of protein instability is not restricted to wine; protein pharmaceutical products also experience undesired aggregation (*12*). Protein aggregation reduces the quality and value of these pharmaceutical products and has motivated researchers with diverse backgrounds and methodologies to study protein stability and subsequent aggregation. The study of the kinetics of aggregation is complicated by the fact that this type of phenomenon is often not first- or second-order but a higher-order reaction (*13*) and is further complicated because the measurement methods such as turbidity reading are nonlinear. Protein unfolding (or loss of tertiary structure) usually precedes non-native aggregation events (aggregation that does not occur within a normally functioning live cell). Understanding the factors that dictate protein unfolding is fundamental to understanding aggregate formation. If it is assumed that the kinetics of aggregation are faster than the kinetics of unfolding and unfolding is the rate limiting step, then studying the simpler unfolding step in isolation

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will provide a better indication of the factors affecting aggregation than studying aggregation itself.

Thermal unfolding of proteins can be treated as a thermodynamic phenomenon. While unfolding can involve multiple intermediates and unfolding steps, the unfolding of small globular proteins, such as grape chitinase and thaumatin-like protein, is usually considered to be a single reversible unfolding event, thus simplifying the mathematics. Differential scanning calorimetry (DSC) is a thermal scanning technique and is a useful method for understanding the thermodynamics of unfolding. The DSC can measure the temperature of maximum apparent heat capacity (T_m), partial molar enthalpy (ΔH_u), and the partial molar heat capacity change ($\Delta C_{p,u}$) directly. The T_m value can be used to qualitatively rank a protein's unfolding propensity (*12*). The rate constant (k_u) can also be calculated from the DSC scans (*13*), and the activation energy E_a can then be calculated using the Arrhenius equation. While this approach relies on thermodynamic equilibrium, both reversible and irreversible unfolding have been analyzed following this approach. Irreversible unfolding of some proteins was assumed to be composed of a reversible followed by an irreversible step (*14*) where the rate of the second step was much greater than the first. This enabled the reaction to be treated as a first-order reaction with Arrhenius behavior (*13*). Aggregation prone and relatively insoluble proteins can make the determination of thermodynamic parameters difficult using DSC thermal scanning (*12*). In this case, k_u can be calculated using accelerated stability testing at elevated temperatures and observing the kinetics of unfolding using techniques such as circular dichroism spectrometry or fluorescence spectrometry using the protein's intrinsic fluorescence. k_u values outside the experimental range can be predicted by extrapolating an Arrhenius plot and in turn can be used to calculate the predicted shelf life. The use of DSC data and accelerated stability approaches to studying the thermodynamics of unfolding and subsequent aggregation kinetics are comprehensively reviewed in ref *12*.

In this article, we used DSC thermal scans to determine which grape proteins are most likely to be responsible for haze formation. Accelerated stability studies were used to calculate the rate constants for unfolding k_u of the haze-forming protein and to provide a predictive model for shelf life versus the storage temperature found in wine cellars through to shipping containers. The relationship between protein unfolding and haze formation in wine was also discussed.

MATERIALS AND METHODS

Purification of Wine Proteins. Chitinase, invertase, and thaumatin-like protein 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 named as described by Van Sluyter et al. (*15*). All proteins were stored as suspensions in ammonium sulfate at −80 °C.

Reversed-Phase HPLC Analysis. Reversed-phase separation of wine concentrate proteins was carried out on an Agilent 1200 series HPLC system (Santa Clara, CA, USA) equipped with a Jupiter C18 5 *μ*m 300A 150 × 2 mm column (Phenomenex, Lane Cove, NSW, Australia). Ten microliters of wine concentrate was injected onto the column. The column was equilibrated at 83% A (0.1% TFA and 8% acetonitrile) and 17% B (80% acetonitrile and 0.1% TFA) for 5 min. Protein elution was achieved by a 7 min gradient from 17% B to 49% B, from 49% B to 57% B from 7 to 15 min, 57% B to 65% B from 15 to 16 min, and then from 65% B to 85% B from 16 to 40 min (percentages shown are v/v). Flow rate and temperature were maintained at 0.2 mL/min and 35 °C, respectively, throughout the run. Peaks were detected at 215 nm, and their identity was assigned by comparison of their retention times to those of purified grape PR-proteins (*7*).
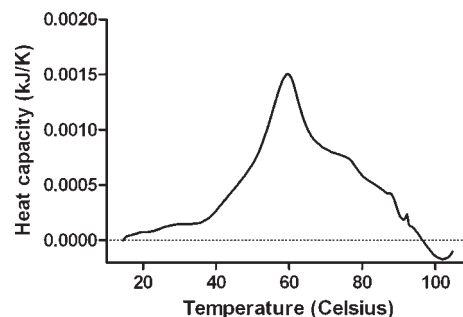
Figure 1. DSC scan of unfined Sauvignon blanc wine protein concentrated by ultrafiltration (10 kDa).

Differential Scanning Calorimetry. Wine concentrate was obtained by concentrating crude Sauvignon blanc wine using the Amicon Ultra 15 10 kDa filter devices. Purified protein samples were adjusted to around 0.5 mg/mL with artificial wine (2 mg/mL malic acid and 12% (v/v) ethanol, pH 3) and then dialyzed overnight at 4 °C with 3× changes of artificial wine.

Differential scanning calorimetry was undertaken with a VP-DSC (MicroCal, Northampton, MA, USA) 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.

Circular Dichroism Spectrometry and the Kinetic Study of Unfolding. Ellipticity was measured using a circular dichroism spectrometer J-815 (Jasco International, Tokyo, Japan) with a Peltier temperature controlled sample holder between the wavelengths of 260−190 nm. Each spectrum is the average of 5 scans. The spectra at set temperatures were measured in an upward series with 10 min for the temperature to stabilize and continuously mixed with a 7 mm magnetic stirrer.

Kinetics of unfolding were measured using the CD spectrometer with a 1 × 1 cm cuvette with magnetic stirrer. Then 2.5 mL of artificial wine was added to the cuvette and the temperature allowed to stabilize. Then 0.2 mL of 0.5 mg/mL chitinase in artificial wine was added to the cuvette and the measurement started. Measurements were taken every 5 s at 222 nm. Mixing was shown to be complete in less than 10 s.

Reversibility of unfolding was measured using the CD spectrometer as described above except that the temperature was set at 20 °C and allowed to stabilize for 5 min, and the measurement taken between 260 and 190 nm. The temperature was then set to 60 °C and allowed to stabilize for 5 min, and the measurement taken. This procedure was repeated three times.

RESULTS AND DISCUSSION

The DSC thermal scan of the unfined Sauvignon blanc wine protein fraction (concentrated 5-fold using a 10 kDa cut off centrifugal filter) showed a complex series of events taking place as the temperature rises (**Figure 1**). There is a leading edge to the main peak, indicating that there is some change taking place from around 40 °C. The main peak is at 61 °C and is likely to correspond with the most abundant species, the thaumatin-like proteins. A second minor peak is at 72 °C, and the uneven baseline after 80 °C is indicative of aggregation taking place. HPLC analysis of the wine protein concentrate confirms that thaumatin-like proteins are the most abundant protein species and that chitinases and other proteins are also present (**Figure 2**).

Thermal scans of purified wine proteins in an artificial wine (composed of 2 g/L malic acid and 12% (v/v) ethanol, pH 3) supplied the T_m values for chitinases, thaumatin-like proteins, and invertase isolated from Sauvignon blanc and Semillon wines (**Table 1**). The protein with the lowest T_m value was chitinase F1 at 55 °C with the thaumatin-like proteins next with T_m values ranging between 56 and 62 °C. Invertase was shown to be
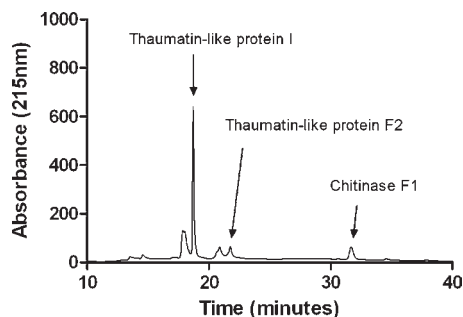
Figure 2. HPLC analysis of unfined Sauvignon blanc wine protein concentrated by ultrafiltration (10 kDa).

Table 1. Melt temperatures for wine proteins measured using differential scanning calorimetry with a scan speed of 1.5 °C per minute

|	protein type	grape variety	T_m (°C)
F1	class IV chitinase	Sauvignon blanc	55
F2	thaumatin-like protein	Sauvignon blanc	56
I	thaumatin-like protein	Sauvignon blanc	62
invertase	vacuolar invertase 1	Sauvignon blanc	81
D1	class IV chitinase	Semillon	55
C	thaumatin-like protein	Semillon	61
B	thaumatin-like protein	Semillon	61
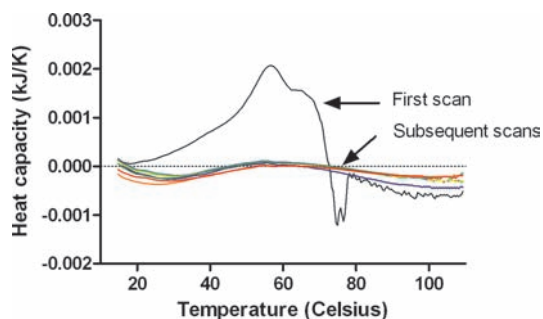
Figure 3. Repeated DSC scans of chitinase F1 from Sauvignon blanc showing a melt temperature of 55 °C, no reversibility of thermal unfolding, and aggregation after unfolding.

relatively stable with a T_m value of 81 °C. This indicates that chitinase is the least stable protein with one of the thaumatin-like proteins also being unstable. Repeated thermal scans of purified wine proteins in an artificial wine were used to differentiate between reversible and irreversible unfolding of the protein. The repeated scan for chitinase F1 from Sauvignon blanc was complex (**Figure 3**). The main peak (T_m) was at 55 °C, but it is preceded by a shift in heat capacity from the start of the scan. There was a dramatic fall in heat capacity after 66 °C, indicative of protein aggregation taking place. Visual observation of the chitinase solution after the experiment showed an obvious haze confirming that aggregation had taken place. The observation of aggregation occurring immediately after thermal unfolding suggests a strong link between unfolding and the initiation of aggregation. The subsequent thermal scans show no thermal effect indicating that the unfolding reaction followed by aggregation reaction was irreversible. Thermal scans of the chitinase D1 purified from Semillon also showed a similar profile, though the visible evidence of aggregation was less pronounced (not shown). Repeated DSC scans of thaumatin-like protein were quite different from those of chitinase and showed a high degree of reversibility of unfolding indicating that on cooling the protein returns to a native or near-native conformation (**Figure 4**). There was
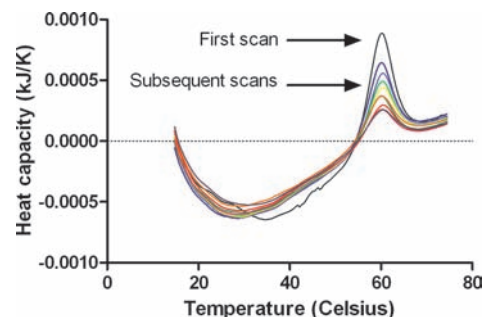
Figure 4. Repeated DSC scans of thaumatin-like protein C from Semillon showing a melt temperature of 61 °C and some reversibility of thermal unfolding.
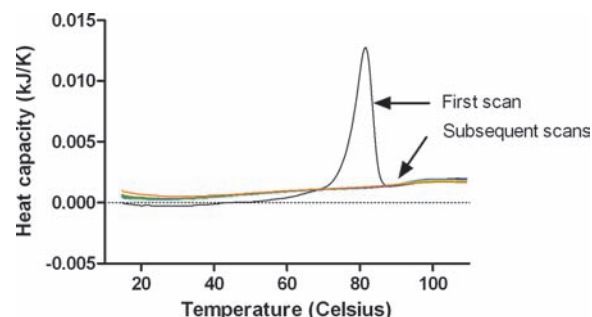

Figure 5. Repeated DSC scans of invertase from Sauvignon blanc showing a melt temperature of 81 °C and no reversibility of thermal unfolding.

also no evidence of aggregation as seen with the chitinases. The thermal scans for the other thaumatin-like proteins isolated from Sauvignon blanc and Semillon also share the profile of thaumatin-like protein C from Semillon, having uniform peaks and no evidence of aggregation, though the different T_m values indicate there was some variation in thermal stability in these closely related proteins. Repeated thermal scans of invertase showed that thermal unfolding of this protein was irreversible without evidence of aggregation (**Figure 5**). The T_m value for invertase was 81 °C, indicating that it was relatively stable compared to the chitinases and thaumatin-like proteins. This result agrees well with observations of others that grape invertase retains significant enzyme activity at pH 4.0 after 10 min at 80 °C (*16*).

The evidence from the DSC experimentation strongly points to chitinase being a major cause of haze formation in Sauvignon blanc and Semillon wine. Chitinase has the lowest T_m value ranking it as the least stable of the grape proteins. The evidence of aggregation occurring during the thermal scanning seen in the scans and visually after the experiment also demonstrates that chitinase has a strong propensity to aggregate. Thaumatin-like protein was the next most likely candidate for haze formation with a T_m value marginally higher than that of chitinase, but the reversibility of unfolding indicates that this protein does not readily form an irreversible aggregate, at least under the conditions of our experiments. Invertase was unlikely to be the cause of protein hazes in wine being relatively stable compared to chitinase and thaumatin-like protein. Subsequent work was focused on chitinase to understand the kinetics of unfolding that precedes aggregation.

The complexity of the chitinase thermal scan makes it unsuitable for deriving thermodynamic information. Aggregation interferes with the end of the peak, making ΔH_u and $\Delta C_{p,u}$ impossible to estimate. One approach would be to repeat the DSC
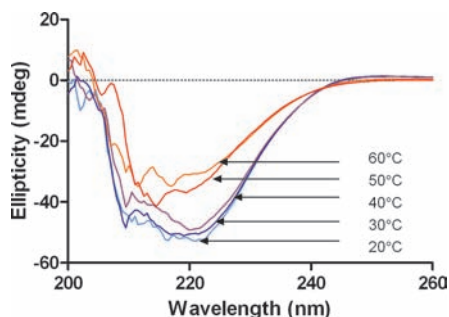
Figure 6. Circular dichroism spectra of the chitinase F1 in artificial wine at different temperatures. Light blue (20 °C), dark blue (30 °C), purple (40 °C), red (50 °C), and orange (60 °C).

analysis at lower protein concentrations to minimize aggregation. However, without dilution, the size of the unfolding peak for chitinase was already at the lower limits for determining ΔH_u and $\Delta C_{p,u}$. The alternative approach to thermal scanning using the DSC is to apply spectrometry to identify unfolding. Circular dichroism spectrometry (CD) is widely used to identify α-helices within protein structures and was suitable for our purposes.

A CD scan of chitinase F1 from Sauvignon blanc at 20, 30, 40, 50, and 60 °C demonstrates that chitinase was rich in α-helices that unwind at elevated temperatures, reducing the protein's ellipticity (**Figure 6**). The artificial wine (malate−ethanol solution) interfered with the CD spectrum below 220 nm; however, immediately above 220 nm was a zone where interference was minimal, and the change in protein ellipticity was measurable, making it suitable for measuring the kinetics of chitinase unfolding.

The kinetics of chitinase unfolding were amenable to measurement in the 42−55 °C temperature range with unfolding half-lives of 0.5 min at 55 °C, 2.5 min at 50 °C, 17 min at 45 °C, and 60 min at 42 °C (see plots for 45, 50, and 55 °C in **Figure 7**). The rate constants (k_u) calculated for 42, 45, 50, and 55 °C enabled the activation energy E_a to be determined as 320 kJ mol^{-1} using the Arrhenius equation. The predicted half-lives of chitinase at temperatures between 10 and 45 °C are shown in **Table 2**. Chitinase was unstable at temperatures that would be experienced by wine in nonrefrigerated storage or transport environments, especially in warmer countries where wine is produced. If the assumption that chitinase unfolding is the rate limiting step in haze formation, then the predicted shelf-temperatures are a useful guide to haze formation in artificial wine and possibly also for commercial unfined or underfined wines. Indeed, the observations by Pocock and colleagues (*17*, *18*) that six out of eight commercial unfined wines stored at 16 to 20 °C developed haze within a year of storage and the other two wines within 3 years, and that all the wines were hazy after storage at 37 °C for a week aligns well with the data presented here.

The extrapolation of the predicted unfolding kinetics to temperatures below 45 °C may be misleading if the mechanism is not a simple rate limiting reversible unfolding step. The average ellipticity of the chitinase at 222 nm varies depending on the temperature (**Figure 6**). This could be interpreted two ways, as differing levels of chitinase unfolding at equilibrium at different temperatures or as different proportions of two unfolding isomers at equilibrium. The structures of related chitinases such as jack bean chitinase (*19*) are known to have multiple α-helices and a tertiary structure that could be amenable to multiple unfolding outcomes with varying amounts of residual structure (**Figure 8A**). Differing levels of unfolding of chitinase is likely to affect its propensity to aggregate. The CD spectra for chitinase at 50 and
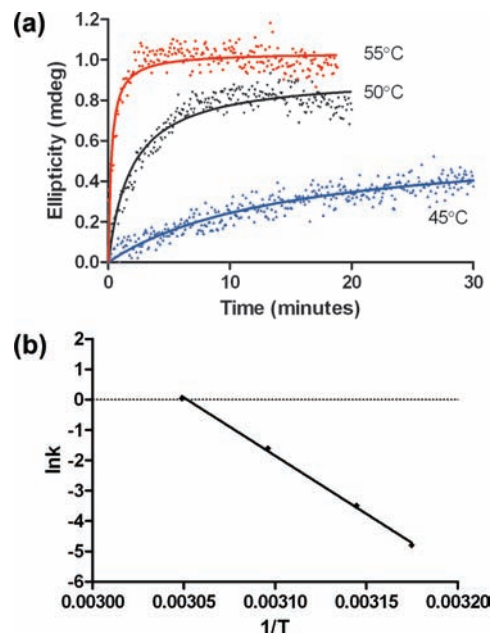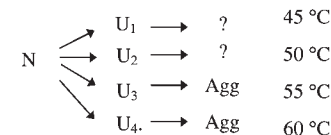

Figure 7. Kinetics of chitinase F1 denaturation, circular dichroism measurement of the α helix structures at 222 nm and corresponding Arrhenius plot. Blue (45 °C), black (50 °C), and red (55 °C).

Table 2. Predicted Half-Life of chitinase in Artificial Wine Based on the Results of the Kinetics of the Unfolding Experiment

temperature (Celsius)	half life
45	17 min
40	1.3 h
35[a]	14 h
30[a]	4.7 days
25[a]	1.3 months
20[a]	1 year
15[a]	9 years
10[a]	100 years

[a] This prediction assumes that unfolding is rate limiting; if aggregation becomes rate limiting, the values shown here underestimate the predicted half-life.



Figure 8. (**A**) Residual structure model based on unfolding kinetics of chitinase F1 (measured by CD) at different temperatures. (**B**) Partially reversible model based on CD reversibility data. N, native structure; U, unfolded structure; Agg, aggregated protein.

60 °C, and for 20, 30, and 40 °C (**Figure 6**) were very similar, indicating that a residual structure model was not supported by these data. The alternative theory that explains the different ellipticity at different temperatures is explained by a reversible step between the native state and the final aggregated form (**Figure 8B**). Reversibility of chitinase unfolding was tested by repeatedly heating chitinase to 60 °C and cooling it to 20 °C, and measuring the ellipticity at each temperature (**Figure 9**). The aggregation reaction was minimized by using a chitinase

Article

J. Agric. Food Chem., Vol. 58, No. 2, 2010   **979**
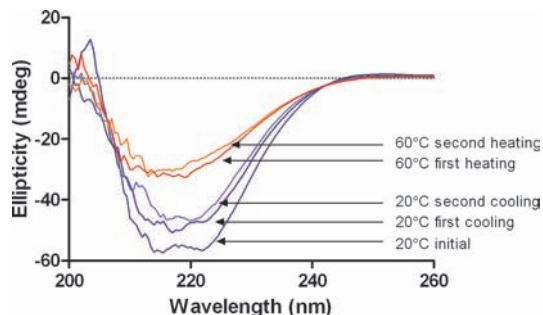
Figure 9. Circular dichroism spectra of chitinase in artificial wine repeatedly heated to 60 °C then cooled to 20 °C. Black (20 °C initial reading), red (heated to 60 °C for 10 min), blue (cooled to 20 °C for the next 10 min), orange (heated to 60 °C for 10 min), and light blue (cooled again to 20 °C).

concentration 50-fold less concentrated than that used in the DSC experiments, reducing the aggregation rate by at least 2500-fold compared to that in the DSC experiment (assuming aggregation is a second order reaction). The results of this work showed that native chitinase (N) thermally treated at 60 °C (U_B) did not return to the native conformation (N) on cooling to 20 °C but to a non-native conformation (U_A) with less ellipticity than native chitinase. Repeated thermal treatment followed by cooling to 20 °C was shown to shift the chitinase between U_A and U_B. This observation explains the different ellipticities at different temperatures and supports a partially reversible model for chitinase unfolding (**Figure 8B**). As U_A and U_B are likely to have different propensities to aggregate (with U_B being more likely to aggregate as it has the least residual structure), the proportion of U_A and U_B will directly impact the aggregation rate. The partially reversible model does impact the accuracy of the shelf life prediction based on kinetics alone (**Table 2**) as the equilibrium constant is not taken into account. The aggregation rate is likely to have a second order (or higher) relationship to the concentration of the aggregation precursor U_B (*20*). This implies that U_B concentration is a function of both the rate constant k_u and the equilibrium constant between U_A and U_B. While unfolding is a critical precursor to aggregation, the kinetics of aggregation (and its visible manifestation haze) may be complex and requires further investigation.

The data described here suggest that chitinase is primarily responsible for heat induced haze in artificial wines and is likely to be responsible for hazes in unfined or underfined Semillon and Sauvignon blanc wines on the basis of the low T_m value and observed post-unfolding aggregation. However, it must be noted that the role of other wine components, such as sulfate, metal ions, and phenolic compounds, was not examined in this study, and these compounds may well modify the behavior of the proteins in authentic wines. Furthermore, it must be remembered that any one protein studied here does not exist in isolation of the other proteins in authentic wines, and interactions between the proteins are likely. Indeed, the observation by Esteruelas and colleagues of multiple proteins in naturally occurring Sauvignon blanc wine precipitates (*9*) is probably due to chitinase inducing aggregation that then strips thaumatin-like protein and other wine proteins out of solution. Identification of the cause of haze formation could influence the technology used to produce heat-stable commercial wines. Current bentonite fining is a nonspecific method of removing proteins from wine or grape juice. Bentonite fining has some negative attributes including dilution of the wine by the bentonite slurry, handling and disposal problems associated with spent bentonite, and quality loss of wine recovered from lees. Selective removal of chitinase from juice or wine using reusable absorbent technology or proteases may result in heat-stable wines still containing thaumatin-like protein and other grape proteins. While the sensory properties of an unfined wine may be similar to bentonite fined wines (*21*), some physical properties such as foam formation in sparkling wine base may well be desirable (*22*).

ABBREVIATIONS USED

DSC, differential scanning calorimeter; CD, circular dichroism; HPLC, high-performance liquid chromatography; T_m, temperature of maximum apparent heat capacity; ΔH_u, partial molar enthalpy; $\Delta C_{p,u}$, partial molar heat capacity; k_u, unfolding rate constant; E_a, activation energy; N, native structure; U, unfolded structure; Agg, aggregated protein.

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Falconer et al.

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