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Review

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Wine Protein Haze: Mechanisms of Formation and Advances in Prevention

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ABSTRACT: Protein haze is an esthetic problem in white wines that can be prevented by removing grape proteins that have survived the winemaking process. The haze-forming proteins are grape pathogenesis-related proteins that are highly stable during winemaking, but some of them precipitate over time and with elevated temperatures. Protein removal is currently achieved by bentonite addition, an inefficient process that can lead to higher costs and quality losses in winemaking. The development of more efficient processes for protein removal and haze prevention requires understanding the mechanisms that are the main drivers of protein instability and the impacts of various wine matrix components on haze formation. This review covers recent developments in wine protein instability and removal and proposes a revised mechanism of protein haze formation.

KEYWORDS: *bentonite alternatives, chitinases, pathogenesis-related proteins, protease, protein aggregation, thaumatin-like protein, wine haze, wine heat instability, wine protein*

INTRODUCTION

In 2012 there were 7.528 million hectares of cultivated grape vines among 92 countries, making grapes the largest fruit crop by land area in the world.[1,2] Furthermore, much value is added in the form of winemaking to over half the world's grapes, with the production of 252 million hectoliters of wine in 2012.[2] The contribution of the wine sector to the world economy in 2013 reached a value of U.S.$277.5 billion,[3] with a large proportion of the wine exported. Thus, a substantial volume of wine is subject to potentially damaging conditions during transportation and storage, such as inappropriate temperature or humidity, that can cause deleterious modifications of the organoleptic features of the wine.[4]

Wine clarity, especially that of white wines (Figure 1), is important to most consumers and is also one of the characteristics that is most easily affected by inappropriate shipping and storage conditions. For this reason, securing wine stability prior to bottling is an essential step of the winemaking process and presents a significant challenge for winemakers. A stable white wine is one that is clear and free from precipitates at the time of bottling, through transport and storage, to the time of consumption. Hazy wine and the presence of precipitates are most commonly caused by three factors: microbial instability, tartrate instability, and protein heat instability.[5] Microbial stability is achieved prior to bottling by sulfur dioxide addition and filtration;[6] tartrate stability is achieved by either cold stabilization, ion exchange resins, or electrodialysis.[7]

Figure 1. Clear white wine and turbid wine caused by protein aggregation.

Protein stability in commercial winemaking is almost always achieved by the addition of bentonite, a clay cation exchanger that binds proteins and removes them from wine through precipitation. Protein-bound bentonite settles loosely to the bottom of wine tanks as lees, which account for around 3−10% of the original wine volume.[8] Wine is recovered from bentonite

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lees through processing using rotary drum vacuum filtration, specialized lees filtration equipment, or centrifugation—processes that are considered laborious and that can potentially degrade wine quality.[8−10] Quality degradation and loss of wine through bentonite usage has been estimated to cost the global wine industry around U.S.$1 billion per year.[11] Other issues and costs related to bentonite use include tank downtime for bentonite treatment, occupational health risks associated with inhalation of bentonite dust and slip hazards induced by bentonite slurry spills, the disposal of hazardous bentonite waste, and bentonite interference with increasingly common membrane-based winemaking technologies.[12] Consequently, winemakers aim to use the minimum amount of bentonite required for protein stability and would welcome the introduction of alternatives with fewer drawbacks than the current practice.

Since the last extensive review on the topic a decade ago,[8] research efforts have been equally divided into the elucidation of protein haze-forming mechanisms, in particular the effects of different wine components, as well as improving bentonite efficiency and finding alternative stabilization strategies. Significant attention has also been paid to developing methods for protein purification, quantification, and identification, as well as predicting wine haze potential (Figure 2).

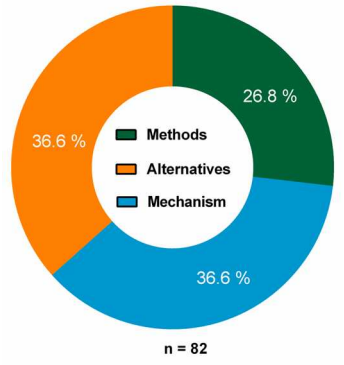

Figure 2. Distribution of peer-reviewed publications on wine protein haze, 2005−2013 (data from Scopus). Methods: papers on the development of methods for purification, quantification, and characterization of proteins, as well as on the prediction of haze potential. Alternatives: papers investigating possible alternatives to bentonite for wine protein stabilization. Mechanism: papers on the elucidation of the mechanism of haze formation in white wines.

This review summarizes recent advances in the knowledge of how protein haze forms in wine, as well as the latest alternatives to bentonite wine protein stabilization. The findings of recent research and the newly proposed mechanisms for haze will be discussed in part I. New alternatives to bentonite will be discussed in part II.

PART I. MECHANISMS OF PROTEIN HAZE FORMATION IN WHITE WINES

Current Model of Haze Formation. The mechanisms associated with haze formation in wines are not well understood and yet str commonly cited as two-stage processrd. In the first stage, wine proteins unfold in response to stimuli such as elevated storage temperatures. Once unfolded, the proteins aggregate and flocculate to form a visible haze.[13] Recent investigations of the proteins associated with haze formation, as well as the roles of other wine components, have enabled the proposed model to be revised into three separate stages described below. The steps include protein unfolding, protein self-aggregation, and aggregate cross-linking.

Haze-Forming Proteins. The isolation and characterization of proteins from white wines have traditionally been difficult tasks due to the presence of grape and yeast proteins as well as their modified versions and degradation products caused by winemaking, which produces a complex protein mixture.[14,15] However, recent advances in techniques for wine protein purification,[16,17] as well as applications of newly developed proteomic techniques,[16,18−25] and the release of the grape genome[26] have significantly improved research capabilities in the identification and quantification of grape and wine proteins.

The most abundant classes of haze-forming proteins that occur in grape (*Vitis vinifera*) juice and white wines are chitinases and thaumatin-like proteins (TLPs).[14,27−29] These proteins are small (<35 kDa) and compact, have globular structures,[30] are positively charged at wine pH, and are tolerant of low pH in juice and wine.[8,31] Other proteins, such as *β*-glucanases, have also been shown to contribute to haze formation,[32,33] although they are much less abundant than chitinases and TLPs in wine and are not extensively studied. A typical electrophoretic profile of grape juice is shown in Figure 3, highlighting that pathogenesis-related (PR) proteins (*β*-1,3-glucanases, chitinases, TLPs, and lipid transfer proteins) are the major protein classes represented. However, haze-forming proteins vary in concentration and composition in ripe grapes and grape juice with cultivar,[34] vintage,[35] disease pressure,[36] and even harvest conditions.[37] The haze-forming proteins have been identified as those that are historically considered to be PR proteins, although they are constitutively expressed during berry ripening and can reach high concentrations regardless of pathogen exposure.[27,38,39] Both chitinases and TLPs have a high number of disulfide bonds that contribute to the highly stable globular structures of these proteins and make them inherently resistant to the enzymatic activity of pathogens.[30,40]

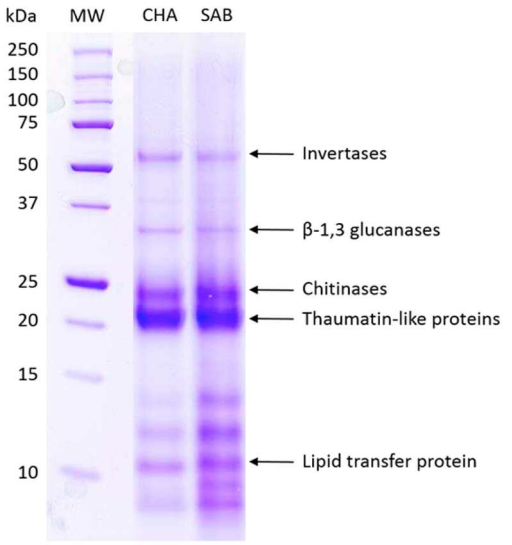

Figure 3. Typical electrophoretic profiles of two unfined grape juices (CHA, Chardonnay; SAB, Sauvignon blanc), with protein band identities assigned by proteomic analysis.[85]

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Non-haze-forming proteins are also present in juice and white wine, including yeast mannoproteins,[41,42] grape invertase,[43,44] and grape cell wall glycoproteins and proteoglycans rich in arabinose and galactose (or arabinogalactan-proteins).[45,46] The presence of these non-haze-forming proteins can also affect white wine quality by stabilizing wine against heat-related protein instability,[47−49] influencing foaming properties in sparkling wines,[43,50] and possibly interacting with aroma compounds.[51,52]

The haze-forming tendencies of proteins isolated from white wines have been shown to depend on their aggregation behavior, and developments in the physical techniques used to characterize this behavior have improved the understanding of protein instability.[53−55]

In wines, different classes of haze-forming proteins have different thermal stabilities, as demonstrated by combinatorial peptide ligand libraries (CPLL) analyses[56] and differential scanning calorimetry (DSC).[57] Chitinases are generally less stable than TLPs and can denature within minutes at temperatures >40 °C, compared to weeks for TLPs under the same conditions[57] (Table 1).

Table 1. Predicted Half-Lives of Chitinases and TLPs in Artificial Wine (Based on Falconer et al.[57])

temperature (°C)	predicted half-lives for chitinases	predicted half-lives for TLPs
50	3 min	20 days
45	17 min	11 weeks
40	1.3 h	13 years
35	14 h	180 years
30	4.7 days	>1000 years
25	1.3 months	>1000 years
20	1 year	>1000 years
15	9 years	>1000 years
10	100 years	>1000 years

The temperature at which proteins unfold can also indicate stability, and recent experiments using differential scanning calorimetry (DSC) analyses have demonstrated differences in the melt temperature of chitinases and TLPs. The physical characteristics and aggregation tendencies of these proteins are given in Table 2. Chitinases unfold at a lower temperature than

Table 2. Summary of General Properties of Chitinases and TLPs

property	chitinases	stable TLPs	unstable TLPs
unfolding temperature	55 °C[57]	61−62 °C[57]	56 °C[57]
aggregate characteristics	visible aggregates (≥1 μm)[54]	microaggregates (<150 nm)[53,54]	visible aggregates (≥1 μm)[30,57]
aggregation tendency	self-aggregate[28,54]	cross-linked with other wine components[53,54]	self-aggregate[30]

TLPs, at 55 and 62 °C, respectively,[57] further indicating that they are less stable. As shown in Figure 4, once unfolded, chitinases did not regain their original structure (renature) after cooling, and this irreversible unfolding (denaturation) was shown to lead to protein aggregation and subsequent precipitation of chitinases.[57]

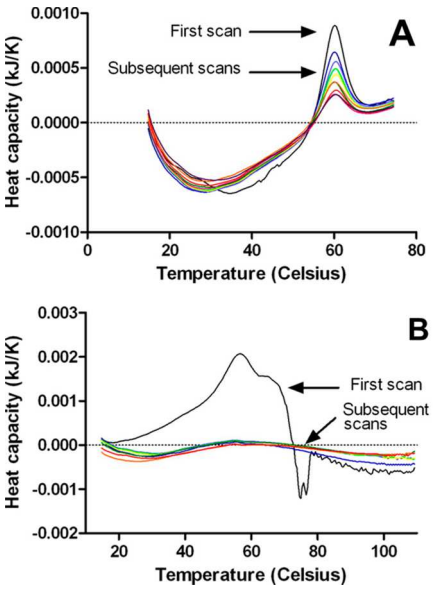

Figure 4. (A) Repeated DSC scans of thaumatin-like protein C from Semillon juice showing a melt temperature of 61 °C and reversibility of thermal unfolding. (B) Repeated DSC scans of chitinase F1 from Sauvignon blanc juice showing a melt temperature of 55 °C, no reversibility of thermal unfolding, and aggregation after unfolding. Reprinted from Falconer et al.[57]

Results from DSC and dynamic light scattering (DLS) experiments have indicated that TLPs generally do not to contribute to the formation of visible aggregates,[28,54] although recent studies have indicated the presence of TLPs in wine hazes.[14,32] This apparent conflict of results most likely relates to the particular isoform of TLP that was used in the experiments. Recent studies have shown that some TLP isoforms will reversibly unfold/refold after heating and cooling, whereas other isoforms will irreversibly unfold (denature) and aggregate. Only the TLP isoforms that denature will participate in haze formation.[28,30,53,57,58]

Crystallography has been used to elucidate the 3D structures of three grape TLP isoforms displaying different hazing potentials and unfolding temperatures (Figure 5).[30] That study demonstrated a high degree of structural similarity among different TLP isoforms. However, a TLP isoform (4JRU) with lower unfolding temperature than the other two (56 vs 62 °C) showed the potential to aggregate upon unfolding in the

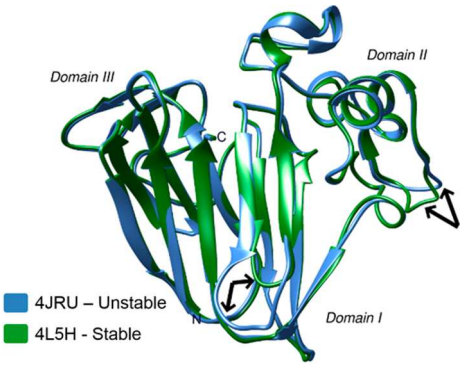

Figure 5. Superposition of the backbone representation of thaumatin-like proteins 4JRU (heat unstable) and 4L5H (heat stable).[30] Arrows indicate differing loops between the two protein isoforms.

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presence of sulfate to form visible haze. The reason for this different hazing potential was attributed to small structural differences related to the conformation of a single loop (located in domain 1) and the amino acid composition of its flanking regions, which could explain some of the variation of hazing potential among wines of similar total protein concentrations.

When proteins unfold and aggregate, the protein type can influence the characteristics of the resulting aggregates. TLPs tend to produce metastable microaggregates (<150 nm at normal wine ionic strength) that are invisible to the naked eye, whereas chitinases can rapidly flocculate and produce clearly visible large aggregates (≥1 μm).[13,54] Model studies have indicated that increasing the concentration of protein in solution will increase the amount of haze produced,[59,60] and yet no such correlation has been shown to exist in wine.[28,31] This is most likely due to other components in wine that can facilitate haze or prevent protein aggregation. For example, most TLP isoforms need to interact with other wine components such as salts or polyphenolics to contribute to visible haze.[53,54] Therefore, the size of TLP aggregates is wine-dependent.[13] The chitinases tested to date form aggregates by themselves after unfolding and cooling, assuming that there is sufficient solution ionic strength to suppress electrostatic repulsion.[54,55] This will be discussed in greater detail in the next section.

Other Wine Components That Contribute to Haze Formation. In addition to differing aggregation behaviors of different wine proteins, other components of wine can also contribute to haze formation. These components include polyphenols, sulfate, formerly indicated as the factor X required for protein haze formation,[61] and polysaccharides in particular, as well as characteristics of the wine matrix such as wine pH and organic acids.[28,52−55,58,62] In the two-stage model of haze formation, proteins first unfold and then aggregate to form a haze, and each of these mechanisms has different drivers. The mechanism of protein unfolding is largely influenced by temperature, with higher temperatures leading to more rapid protein unfolding.[13,28,53,54,57,58] However, this does not fully explain the gradual haze formation that can occur during wine storage, indicating that drivers in addition to temperature play a role.

The mechanism of protein aggregation differs for different protein classes,[28] is influenced by a number of factors, and is likely to be affected by other components present in the wine matrix.[54,58] When proteins unfold, they expose hydrophobic binding sites that are generally buried in the core of the proteins, and more hydrophobic proteins tend to cause hazes more easily (Table 2). This suggests that the aggregation stage of haze formation is likely to be driven by hydrophobic interactions,[63] as recently confirmed by structural studies on TLPs.[30]

Haze-forming proteins also have a net positive charge at wine pH, and this can prevent protein aggregation and haze formation in model systems due to electrostatic repulsion. In white wines the presence of charged ions in solution increases the ionic strength, thus decreasing electrostatic repulsion among protein molecules, so that proteins aggregate upon unfolding and subsequent exposure of hydrophobic protein binding sites.[64] The presence of other charged molecules, particularly sulfates,[61] can also influence protein aggregation, as has been demonstrated with chitinases and, to a lesser extent, TLPs using DLS.[54] In that study model wine containing only chitinases did not form a haze upon heating, whereas increasing sulfate concentration in the matrix led to a dramatic increase in chitinase aggregation. The effect of sulfate on protein aggregation was beyond that which could be attributed to its contribution to ionic strength alone. It was therefore suggested that sulfate not only allows aggregation to occur by suppressing electrostatic repulsion but also allows, if not promotes, hydrophobic interaction-driven aggregation through kosmotropic effects; sulfate anions interact with the hydration water that weakens hydrogen bonding between water and proteins, thus favoring salting-out and aggregation.[54]

In combination with ionic strength and temperature considerations, wine pH can prevent some wines from hazing while promoting haze in others.[55,65] Changes to wine pH have been shown to induce minor protein conformational changes that can change the temperature at which wine begins to show turbidity.[66] In that work, Erbaluce wines with lower pH (3.0) did not form a visible haze upon heating to 80 °C, whereas at higher pH (pH 3.30) the wine became hazy when heated to 60 °C.[66] Organic acids have also been attributed a stabilizing effect in wine protein stability.[65] The authors stated that at wine pH organic acids interact electrostatically with the wine proteins and speculated that this interaction would prevent wine compounds of phenolic nature to interact with the wine proteins and thus facilitate haze.[65]

The impact of wine pH on protein stability varies with protein type. Variations in wine pH from 2.5 to 4.0 at room temperature were sufficient to disrupt the native state of chitinases, resulting in the exposure of hydrophobic binding sites that eventually facilitated protein aggregation.[58] Conversely, TLPs and invertases were stable under the same conditions, further demonstrating the comparative instability and haze-forming potential of chitinases compared with stable TLP isoforms.

Polyphenols can also contribute to the aggregation and precipitation of wine proteins, and this can be due to the formation of hydrogen bonds or most likely to hydrophobic interactions.[65,67−69] A wide range of polyphenols have been identified from naturally precipitated proteins in Sauvignon blanc wines that were stored below 30 °C.[70] These include condensed tannins from grapes that are known to readily bind to proteins. Spiking experiments have indicated that polyphenols actively aggregate and precipitate wine proteins at room temperature,[63] most likely due to cross-linking protein aggregates forming larger aggregates that are visible to the naked eye. Cross-linking of proteins with nonprotein molecules includes both covalent and noncovalent interactions. However, because white wines are generally produced under nonoxidative conditions, there are probably few cases of phenolic compounds oxidizing to highly reactive quinones and covalently cross-linking proteins.

Therefore, the growth in size of protein self-aggregates seems also attributable to the cross-linking action of other matrix components. This will be discussed further in the next section. Elevated temperatures can increase polyphenol−protein interactions and aggregation because increased temperatures will increase the number of protein hydrophobic sites that are exposed, as well as the intensity of the hydrophobic interactions.[67] The exposure of hydrophobic sites also differs in magnitude and consequence depending on protein type.[63] Polysaccharides can also influence haze formation, although reports vary between stabilizing proteins against aggregation[41,47,49] to inducing haze formation.[59] This variation may be due to differences in the measured polysaccharide/protein

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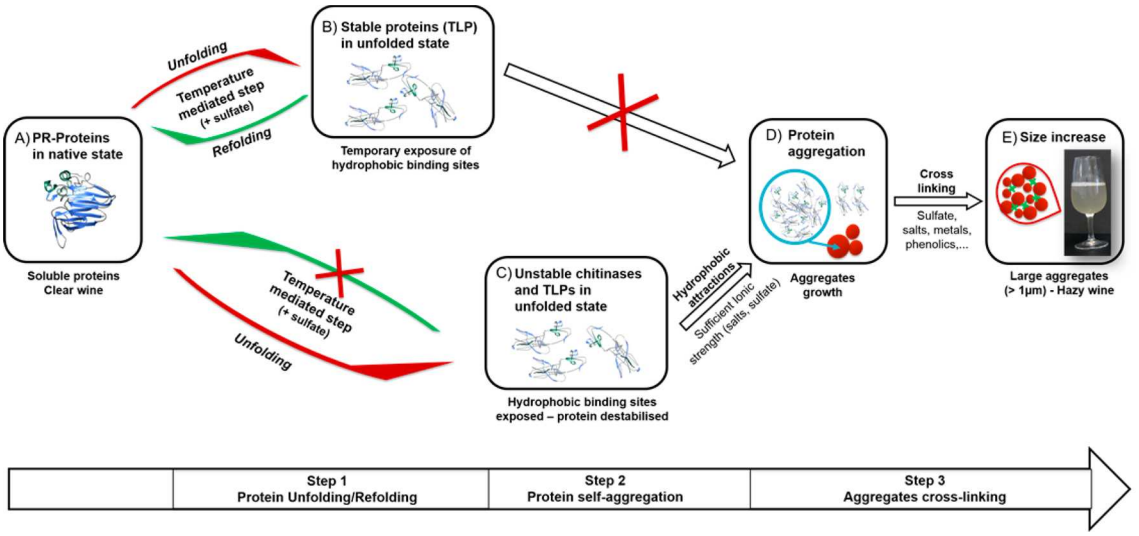

Figure 6. Revised unfolding and aggregation mechanisms of heat-unstable proteins in wine.

ratio or in the type of protein, such as TLP isoform, used in the analysis.[53] Gazzola et al. showed that wine polysaccharides played a crucial role in haze formation of TLPs, whereas this was not the case for chitinases. In general, it seemed that the type of protein was always more important than its interaction with polysaccharides.[53]

Another aspect investigated was the theory that denatured proteins (e.g., chitinases) could interact with otherwise soluble proteins (e.g., TLPs) in a coprecipitation mechanism (protein−protein interaction). Although this theory has not been comprehensively disproven in real wines, it has been shown to be insignificant in model wine and one Sauvignon blanc.[28]

Revised Mechanism of Protein Haze Formation. Haze formation is caused by the unfolding and aggregation of grape-derived wine proteins and can lead to precipitation. The current model of wine protein aggregation indicates that protein unfolding and aggregation are separate events, as demonstrated through DLS experiments.[13] Elucidating the mechanisms of haze formation has involved different analytical approaches characterizing naturally forming hazes from wine[28,70] as well as heat-induced haze from real wines.[14,71,72] Heat trials have indicated that proteins can unfold as wine is heated, although the wine becomes hazy only after cooling.[13] The fractionation of heat-unstable wines into their component parts, such as proteins, polysaccharides, and phenolics, and the heat aggregation behavior studied via reconstitution experiments in model or real wines have also improved the knowledge of the haze-forming mechanism.[28,53,54,61,63] Other significant advancements in understanding the mechanisms of wine haze include (i) the finding that sulfate plays an important role in hazing;[54,61] (ii) the explanation of the role of nonproteinaceous wine components, particularly ionic strength,[54,55] pH,[58,66] organic acids,[65] and phenolic compounds;[53,63,70] (iii) the development of an efficient protein purification method;[17] (iv) the release of the grape genome[26] and greater accessibility of proteomic techniques for protein characterization; (v) the discovery that TLPs and chitinases have different unfolding temperatures and unfolding/aggregation behavior;[57] and (vi) the solution of the crystal structure of thaumatin-like proteins.[30] On the basis of these advancements, a new model of haze formation has been proposed (Figure 6).

Immediately following winemaking and clarification, wine proteins are stable and folded in their native state, and the wine is clear. The first stage of haze formation involves the unfolding of these proteins in response to elevated storage temperatures, revealing the hydrophobic binding sites that are generally buried in the core of the proteins.[63] For TLPs this mechanism has recently been elucidated.[30] It appears that unstable TLPs have an exposed loop stabilized by a disulfide bridge that, if destabilized via heat, can expose the neighboring protein region (Figure 7).

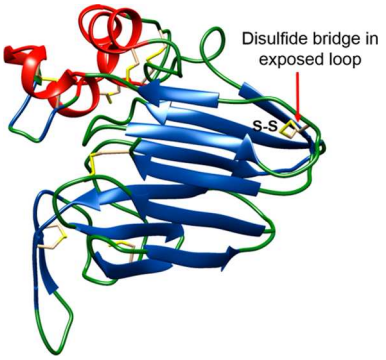

Figure 7. Backbone representation of the heat-unstable thaumatin-like protein 4JRU.[30] Disulfide bonds are yellow. The arrow indicates an exposed disulfide bond that could be susceptible to reduction by heat and sulfites.

In heat-unstable TLPs (e.g., 4JRU) the neighboring region that becomes exposed upon reduction of the disulfide bridge located in the exposed loop is hydrophobic; thus, protein aggregation can occur under conditions that favor disulfide bond reduction, such as heating in the presence of sulfites.[30] Conversely, stable TLP does not precipitate due to the hydrophilic nature of exposed regions that prevent protein aggregation and allow refolding upon cooling.[30,57] In the second stage of haze formation unstable proteins begin to self-aggregate via hydrophobic interactions. At this stage wine components able to modify the ionic strength of the solution as salts and sulfate can favor the binding of the unfolded proteins, further promoting protein aggregation.[63] This is particularly the

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case with TLPs. In the third and final stage of aggregation the protein aggregates gradually become cross-linked due to the actions of sulfates and polyphenols. Proteins form increasingly larger aggregates until they reach a size that makes them visible to the naked eye ($\geq$1 μm) and eventually precipitate.[13,54,55] The presence of sulfate and salts can also neutralize protein net charges and reduce the natural electrostatic repulsion between similarly charged proteins, whereas polyphenols are more likely to cross-link protein aggregates via hydrophobic interactions. From this mechanism, the role of sulfate as a key wine matrix component for the formation of haze becomes apparent. Sulfate can potentially participate in each of the three stages of haze formation by modifying the melt temperature of proteins in stage 1, by screening the exposed protein charges in stage 2, thus favoring aggregation of unfolded proteins,[28,54] and by mediating the aggregation of small protein aggregates probably through a cross-linking action at stage 3 to modulate the final haze formed.[54]

Following the new insights into haze-forming mechanisms and protein stability, the next stage of research will focus on developing a new test for assessing the haze potential of white wines. The conditions used in the current test, known as the "heat test",[73] may overestimate the risk that a particular wine will haze by denaturing both haze-forming and non-haze-forming proteins. This can overpredict the amount of bentonite needed to stabilize the wine, leading to less cost-effective winemaking practices. Understanding the mechanisms of haze formation and the structures of the proteins involved can also lead to the development of new strategies for haze prevention in white wines.

PART II: BENTONITE ALTERNATIVES

Strategies for Wine Haze Prevention. Considering the mechanisms of wine protein haze formation, there are several possible strategies for preventing wine haze that would either reduce or eliminate the need for bentonite. These include (i) decreasing the ionic strength of the wine; (ii) decreasing the polyphenol concentrations in wine; (iii) stabilizing wine proteins against thermal unfolding; (iv) disrupting hydrophobic protein−protein interactions; (v) degrading wine proteins enzymatically after heat treatment; and (vi) using alternative adsorbents or ultrafiltration to remove proteins.

Strategies 1 and 2 are most likely impractical from a wine sensory quality perspective. Although it is possible to either decrease the ionic strength of wine or remove polyphenols using existing industrial-scale electrodialysis, ion exchange, and fining technologies, these techniques would dramatically change wine sensory attributes. Strategies 3 and 4 are potentially related in practice, as the promotion or addition of specific glycoproteins/proteoglycans/polysaccharides, including specific yeast mannoproteins, could both stabilize wine proteins and interfere with hydrophobic protein−protein interactions.[55] Protein stabilization with polysaccharides such as pectin and carrageenan is well established in other beverages such as milk[74] and beer, and the ability of a yeast mannoprotein to stabilize wine proteins was attributed specifically to the glycan portion of the proteoglycan.[49] However, in both cases, it is unclear at what point protection against haze formation by polysaccharides occurs: do the polysaccharides protect against denaturation or, once proteins are denatured, protect against aggregation? An unexplored alternative to polysaccharides that would implement strategy 4 would be the addition of surfactants to wines to prevent protein−protein interactions. Surfactants such as polysorbates are commonly used in processed foods and beverages to stabilize emulsions. However, their use in wine is not currently permitted, they might not be acceptable to consumers, and they could negatively affect foam properties in sparkling wines.

The most promising strategies for developing bentonite alternatives are strategy 5, the potential to degrade wine proteins with enzymes, and strategy 6, the potential for developing novel fining agents. The use of enzymes and novel fining agents as bentonite alternatives will be discussed in more detail in the following sections.

Protein Degradation Using Enzymes. Degrading haze-forming proteins in wine with enzymes is a particularly appealing alternative to bentonite because it minimizes wine volume loss and aroma stripping. Ideally, effective enzymes would be added to grape juice or ferment without the need for later removal, such as with pectinases and glucanases.[75] The degradation products of grape proteins may also be utilized by yeast as nitrogen sources, potentially reducing the frequent need for nitrogen additions (as diammonium phosphate) and improving wine aroma quality.[76,77] There are two types of enzymatic activity relevant to wine protein degradation: the hydrolysis of peptide bonds by proteases and the reduction of disulfide bonds by protein disulfide reductases. Proteases catalyze peptide bond hydrolysis through nucleophilic attack induced either by an amino acid side chain of the protease, such as for cysteine and serine proteases, or by an activated water molecule, such as for metalloproteases and aspartic proteases.[78] Proteolytic enzymes are routinely used in the beverage industry, for example, papain, a cysteine protease from papaya that is used in beermaking,[79] and are therefore a viable option for use in winemaking. Protein disulfide reductases could, theoretically, destabilize and precipitate haze-forming proteins during winemaking via the enzymatic reduction of disulfide bonds, because the chemical reduction of disulfide bonds has been shown to facilitate the unfolding of these proteins.[63] However, there have been no published examples of protein disulfide reductases being active under wine conditions. For this reason, the search for wine-relevant enzymes to degrade haze-forming proteins has focused on proteases and, at least since the 1950s, researchers have tried to find proteases that destroy haze-forming wine proteins under winemaking conditions.[80] The difficulty in using proteases for specifically degrading haze-forming proteins in wine is associated with the stability of the proteins in wine-like conditions. Chitinases and TLPs are characteristically highly resistant to proteases in their native state due to their rigid peptide backbone structure[72,81,82] and so can tolerate the endogenous proteases that degrade many grape proteins during crushing and pressing.[29] Grape TLPs, as with PR proteins from other plant species, have been found to be resistant to many different types of proteases and may even inhibit the activity of some proteases.[83,84]

Marangon et al.[85] developed a promising new protease treatment that involves heating grape juice in the presence of a heat-tolerant protease prior to fermentation to produce wine that is free from haze-forming proteins. When juice is heated, the proteins unfold and thus become susceptible to enzymatic activity. The possible drawbacks of exposing grape juice or wine to elevated temperatures are the requirements of high energy inputs and the negative sensory implications,[86−89] even though it has been shown that when applied with care the potential negative sensory changes can be contained, as well as the energy requirements by optimizing the temperature and

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treatment time.[85] Previous research has focused on the ideal temperature and time of heating required to unfold haze-forming proteins without destroying flavor and aroma compounds.[90]

The method of Marangon et al.[85] involved rapidly heating grape juice to 75 °C for 1 min using flash pasteurization. This technique also required the addition of a protease that is active at the low pH of grape juice and white wines (pH 2.9−3.5) and at 75 °C. Aspergilloglutamic peptidase (AGP), known commercially as Proctase and formerly known as Aspergillopepsin II, was found to be active at 50−75 °C.[85] Adding AGP to clarified grape juice prior to flash pasteurization and fermentation resulted in wines that were heat stable and almost completely free from haze-forming proteins. Chemical and sensory results indicated that there were no significant changes to the main physicochemical parameters or wine preference.[85] This combination treatment of protease addition with flash pasteurization has been shown to be effective at industrial scale,[91] and the use of AGP in wine has recently been approved for Australian winemaking.[92] The cost of this treatment compared favorably to bentonite treatment,[85] making it a potentially cost-effective and commercially viable bentonite alternative.

Other proteases are also currently being investigated that are active at winemaking temperatures and are specific against grape haze-forming proteins. Recent investigations have focused on grape pathogens for specificity against PR grape proteins. Plant PR proteins continuously evolve ways to inhibit pathogen growth, and pathogens continuously evolve ways to counteract the inhibitory effects of PR proteins.[93] Elite grape cultivars have been clonally propagated for centuries; therefore, it is likely that pathogens have evolved proteases to destroy PR proteins encoded by ancient grape genes. The juice of grapes infected with *Botrytis cinerea* was found to have significantly lower concentrations of PR proteins than juice from healthy grapes,[36,94,95] whereas the opposite was true in grapes infected with other pathogens such as powdery mildew.[36] One particular protease from *B. cinerea*, BcAP8, has proven to be effective against grape chitinases during juice fermentation without the need for heating.[96] When BcAP8 was added to juice prior to fermentation, the resulting wines produced significantly less heat-induced protein haze than wines made without BcAP8.

Other potential sources of proteases that are active at wine pH include endogenous winemaking sources such as grapes, yeasts, and bacteria, because protein hydrolysis is known to occur during winemaking.[15,97,98] Endogenous grape (*V. vinifera*) proteases including both cysteine and serine proteases[99] have been found in berries and leaves,[88,100,101] although they are generally not well characterized.[102−106] Grape proteases are active at optima from pH 2 to 2.5 and from 60 to 70 °C,[99,107] and the protease activity is generally short-lived after pressing,[88,100] with few exceptions.[99]

Acid-tolerant yeasts and spoilage microbes have been found to secrete proteases at wine pH, although the secreted protease activity was not sufficient to stabilize wine.[108−114] An extracellular pepsin-like aspartic acid protease of 72 kDa was characterized from a *Saccharomyces cerevisiae* isolate,[115] one of the few isolates that secrete protease activity.[111,115] The secreted yeast protease activity discovered by Younes et al.[115,116] was active at wine pH during grape juice fermentation, although it did not affect grape PR proteins until after fermentation when the wine was incubated at 38 °C for prolonged periods. Nonetheless, the discovery of a secreted protease from a *S. cerevisiae* isolate demonstrates that proteolytic activity can occur prior to autolysis of the cell and the release of a vacuolar acid protease, which is a previously established mechanism of protease activity arising from yeast in wine.[117] The isolate that secretes protease activity is not currently used commercially in winemaking,[115] although it could be used as a tool to develop new industrial wine yeast strains that secrete protease.

Novel Fining Agents. Many novel fining agents and other protein removal techniques with the potential to replace bentonite have been explored in recent years. These include seaweed polysaccharides, chitin, zirconium dioxide, and packed-bed cation exchangers, as well as ultrafiltration techniques. Novel fining agents must meet several criteria to effectively compete with bentonite. They must be cost-effective and nontoxic and must not degrade wine quality.

Negatively charged polysaccharides extracted from seaweeds are a potential class of bentonite alternatives.[118] Carrageenan is a food grade polysaccharide that is extracted from red seaweed and is currently used for protein stabilization in the beer industry.[118] It has been shown to be effective in stabilizing white wines at low addition rates (125−250 mg/L), using only one-third or less of the bentonite concentration required.[119] Carrageenan has been found to produce no deleterious sensory impacts compared to bentonite-treated wines.[120] Although those results are promising, residual amounts of carrageenan in treated wines can potentially induce haze formation,[118,119] and this is likely to restrict commercial viability. Another potential protein-adsorbing polysaccharide is chitin. Chitin is a component of crustacean exoskeletons and, alongside its derivatives, such as chitosan, is widely used in industrial processes, including as a thickening agent for processed foods and in pharmaceuticals.[121] The structure of chitin also makes it selective for binding to chitinases, and in-line systems containing chitin can be effective in removing these particular PR proteins from wine,[122] even if its activity in stabilizing wine has been recently challenged.[123] Chitin could potentially have serious sensory impacts, however, by removing favorable wine components such as positive aroma compounds.[124]

The ultimate objective of novel fining agents and protein-adsorbing materials for the wine industry is to achieve wine stabilization using in-line applications with minimum wine loss and no extra processing steps. Zirconium dioxide (ZrO_2) is a readily available protein-adsorbing material that can be regenerated and thus reused.[125,126] ZrO_2 has shown the potential to remove haze-forming proteins when tested in continuous and batch-wise application both during and post fermentation.[12,127,128] However, despite ZrO_2 showing promise and the ability to be regenerated with a simple washing procedure,[129] issues with the flow rates and high dosages required limit its commercial viability.

Ultrafiltration can also be effective in removing haze-causing proteins, although the process is not selective and can remove other, desirable, wine components including polysaccharides.[130] This application is not necessarily viable at winery-scale due the expense of equipment. Packed-bed cation-exchangers could improve process efficiency, and their effectiveness in removing wine proteins has been demonstrated,[131] but they have not as yet been adopted for protein removal.

The mechanisms of wine haze formation have been revised from the two-stage model to a three-stage model and now include protein unfolding, protein aggregation, and the cross-linking of aggregates to form a visible haze. Chitinases and

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TLPs are the most important proteins involved in wine haze formation, and other wine components such as sulfates and polyphenols, as well as wine pH, can influence protein aggregation in the second and third stages. This improved understanding of the mechanisms of haze formation will allow the development of better predictive tools for haze potential and more targeted techniques to prevent hazes forming in bottled white wines. Recent advances in the prevention of haze formation have led to the development of a new bentonite alternative that utilizes a heat-tolerant protease in combination with flash pasteurization. Further investigations into more efficient stabilization strategies, possibly by utilizing proteases that are active at winemaking temperature, will ultimately benefit winemakers worldwide.

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