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1 Wine Protein Haze: Mechanisms of Formation and Advances in 2 Prevention

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

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

21 ■ INTRODUCTION

22 In 2012 there were 7.528 million hectares of cultivated grape
 23 vines among 92 countries, making grapes the largest fruit crop
 24 by land area in the world.^{1,2} Furthermore, much value is added
 25 in the form of winemaking to over half the world's grapes, with
 26 the production of 252 million hectoliters of wine in 2012.² The
 27 contribution of the wine sector to the world economy in 2013
 28 reached a value of U.S.\$277.5 billion,³ with a large proportion
 29 of the wine exported. Thus, a substantial volume of wine is
 30 subject to potentially damaging conditions during trans-
 31 portation and storage, such as inappropriate temperature or
 32 humidity, that can cause deleterious modifications of the
 33 organoleptic features of the wine.⁴

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



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

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

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

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

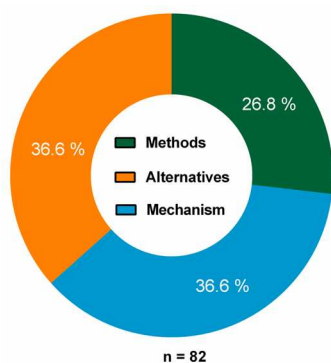


Figure 2. Distribution of peer-reviewed publications on wine protein haze, 2005–2013 (data from Scopus). Methods: papers on the development of methods for purification, quantification, and characterization of proteins, as well as on the prediction of haze potential. Alternatives: papers investigating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

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

85 ■ PART I. MECHANISMS OF PROTEIN HAZE 86 FORMATION IN WHITE WINES

87 **Current Model of Haze Formation.** The mechanisms
88 associated with haze formation in wines are not well
89 understood and yet str commonly cited as two-stage processrd.
90 In the first stage, wine proteins unfold in response to stimuli
91 such as elevated storage temperatures. Once unfolded, the
92 proteins aggregate and flocculate to form a visible haze.¹³
93 Recent investigations of the proteins associated with haze

formation, as well as the roles of other wine components, have
94 enabled the proposed model to be revised into three separate
95 stages described below. The steps include protein unfolding,
96 protein self-aggregation, and aggregate cross-linking.⁹⁷

Haze-Forming Proteins. The isolation and character-
98 ization of proteins from white wines have traditionally been
99 difficult tasks due to the presence of grape and yeast proteins as
100 well as their modified versions and degradation products caused
101 by winemaking, which produces a complex protein mixture.^{14,15}
102 However, recent advances in techniques for wine protein
103 purification,^{16,17} as well as applications of newly developed
104 proteomic techniques,^{16,18–25} and the release of the grape
105 genome²⁶ have significantly improved research capabilities in
106 the identification and quantification of grape and wine proteins.
107

The most abundant classes of haze-forming proteins that
108 occur in grape (*Vitis vinifera*) juice and white wines are
109 chitinases and thaumatin-like proteins (TLPs).^{14,27–29} These
110 proteins are small (<35 kDa) and compact, have globular
111 structures,³⁰ are positively charged at wine pH, and are tolerant
112 of low pH in juice and wine.^{8,31} Other proteins, such as β -
113 glucanases, have also been shown to contribute to haze
114 formation,^{32,33} although they are much less abundant than
115 chitinases and TLPs in wine and are not extensively studied. A
116 typical electrophoretic profile of grape juice is shown in Figure
117 3, highlighting that pathogenesis-related (PR) proteins (β -1,3-
118

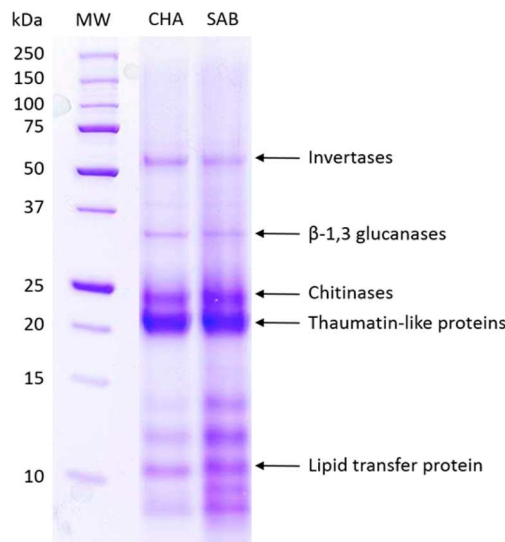


Figure 3. Typical electrophoretic profiles of two unfined grape juices (CHA, Chardonnay; SAB, Sauvignon blanc), with protein band identities assigned by proteomic analysis.⁸⁵

glucanases, chitinases, TLPs, and lipid transfer proteins) are the
119 major protein classes represented. However, haze-forming
120 proteins vary in concentration and composition in ripe grapes
121 and grape juice with cultivar,³⁴ vintage,³⁵ disease pressure,³⁶
122 and even harvest conditions.³⁷ The haze-forming proteins have
123 been identified as those that are historically considered to be
124 PR proteins, although they are constitutively expressed during
125 berry ripening and can reach high concentrations regardless of
126 pathogen exposure.^{27,38,39} Both chitinases and TLPs have a
127 high number of disulfide bonds that contribute to the highly
128 stable globular structures of these proteins and make them
129 inherently resistant to the enzymatic activity of pathogens.^{30,40}
130

131 Non-haze-forming proteins are also present in juice and
 132 white wine, including yeast mannoproteins,^{41,42} grape invertase,^{43,44}
 133 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
 136 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
 139 with aroma compounds.^{51,52}

140 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}

145 In wines, different classes of haze-forming proteins have different thermal stabilities, as demonstrated by combinatorial peptide ligand libraries (CPLL) analyses⁵⁶ and differential scanning calorimetry (DSC).⁵⁷ Chitinases are generally less stable than TLPs and can denature within minutes at 150 temperatures >40 °C, compared to weeks for TLPs under the same conditions⁵⁷ (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

152 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

158 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

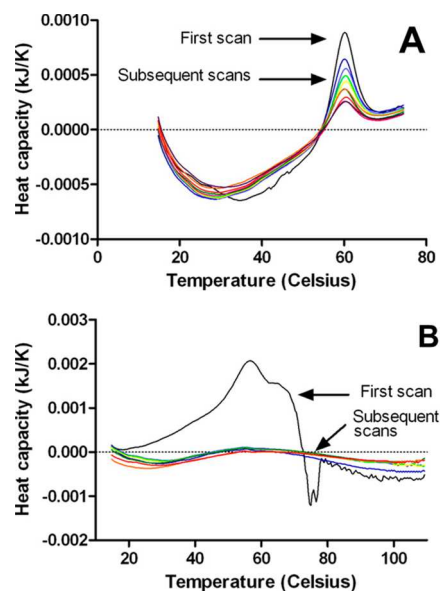


Figure 4. (A) Repeated DSC scans of thaumatin-like protein C from Semillon juice showing a melt temperature of 61 °C and reversibility of thermal unfolding. (B) Repeated DSC scans of chitinase F1 from Sauvignon blanc juice showing a melt temperature of 55 °C, no reversibility of thermal unfolding, and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

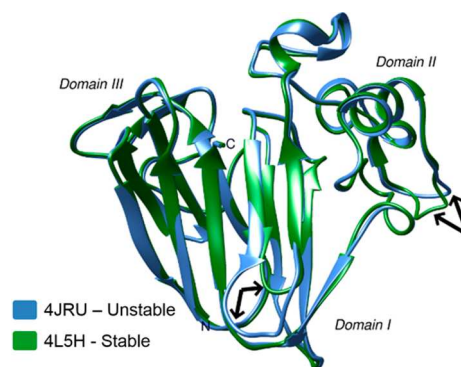


Figure 5. Superposition of the backbone representation of thaumatin-like proteins 4JRU (heat unstable) and 4L5H (heat stable).³⁰ Arrows indicate differing loops between the two protein isoforms.

182 presence of sulfate to form visible haze. The reason for this
183 different hazing potential was attributed to small structural
184 differences related to the conformation of a single loop (located
185 in domain 1) and the amino acid composition of its flanking
186 regions, which could explain some of the variation of hazing
187 potential among wines of similar total protein concentrations.
188 When proteins unfold and aggregate, the protein type can
189 influence the characteristics of the resulting aggregates. TLPs
190 tend to produce metastable microaggregates (<150 nm at
191 normal wine ionic strength) that are invisible to the naked eye,
192 whereas chitinases can rapidly flocculate and produce clearly
193 visible large aggregates ($\geq 1 \mu\text{m}$).^{13,54} Model studies have
194 indicated that increasing the concentration of protein in
195 solution will increase the amount of haze produced,^{59,60}
196 yet no such correlation has been shown to exist in wine.^{28,31}
197 This is most likely due to other components in wine that can
198 facilitate haze or prevent protein aggregation. For example,
199 most TLP isoforms need to interact with other wine
200 components such as salts or polyphenolics to contribute to
201 visible haze.^{53,54} Therefore, the size of TLP aggregates is wine-
202 dependent.¹³ The chitinases tested to date form aggregates by
203 themselves after unfolding and cooling, assuming that there is
204 sufficient solution ionic strength to suppress electrostatic
205 repulsion.^{54,55} This will be discussed in greater detail in the
206 next section.

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

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

233 Haze-forming proteins also have a net positive charge at wine
234 pH, and this can prevent protein aggregation and haze
235 formation in model systems due to electrostatic repulsion. In
236 white wines the presence of charged ions in solution increases
237 the ionic strength, thus decreasing electrostatic repulsion
238 among protein molecules, so that proteins aggregate upon
239 unfolding and subsequent exposure of hydrophobic protein
240 binding sites.⁶⁴ The presence of other charged molecules,
241 particularly sulfates,⁶¹ can also influence protein aggregation, as
242 has been demonstrated with chitinases and, to a lesser extent,
243 TLPs using DLS.⁵⁴ In that study model wine containing only
244 chitinases did not form a haze upon heating, whereas increasing

sulfate concentration in the matrix led to a dramatic increase in
245 chitinase aggregation. The effect of sulfate on protein
246 aggregation was beyond that which could be attributed to its
247 contribution to ionic strength alone. It was therefore suggested
248 that sulfate not only allows aggregation to occur by suppressing
249 electrostatic repulsion but also allows, if not promotes,
250 hydrophobic interaction-driven aggregation through kosmo-
251 tropic effects; sulfate anions interact with the hydration water
252 that weakens hydrogen bonding between water and proteins,
253 thus favoring salting-out and aggregation.⁵⁴ 254

In combination with ionic strength and temperature
255 considerations, wine pH can prevent some wines from hazing
256 while promoting haze in others.^{55,65} Changes to wine pH have
257 been shown to induce minor protein conformational changes
258 that can change the temperature at which wine begins to show
259 turbidity.⁶⁶ In that work, Erbaluce wines with lower pH (3.0)
260 did not form a visible haze upon heating to 80 °C, whereas at
261 higher pH (pH 3.30) the wine became hazy when heated to 60
262 °C.⁶⁶ Organic acids have also been attributed a stabilizing effect
263 in wine protein stability.⁶⁵ The authors stated that at wine pH
264 organic acids interact electrostatically with the wine proteins
265 and speculated that this interaction would prevent wine
266 compounds of phenolic nature to interact with the wine
267 proteins and thus facilitate haze.⁶⁵ 268

The impact of wine pH on protein stability varies with
269 protein type. Variations in wine pH from 2.5 to 4.0 at room
270 temperature were sufficient to disrupt the native state of
271 chitinases, resulting in the exposure of hydrophobic binding
272 sites that eventually facilitated protein aggregation.⁵⁸ Con-
273 versely, TLPs and invertases were stable under the same
274 conditions, further demonstrating the comparative instability
275 and haze-forming potential of chitinases compared with stable
276 TLP isoforms. 277

Polyphenols can also contribute to the aggregation and
278 precipitation of wine proteins, and this can be due to the
279 formation of hydrogen bonds or most likely to hydrophobic
280 interactions.^{65,67–69} A wide range of polyphenols have been
281 identified from naturally precipitated proteins in Sauvignon
282 blanc wines that were stored below 30 °C.⁷⁰ These include
283 condensed tannins from grapes that are known to readily bind
284 to proteins. Spiking experiments have indicated that poly-
285 phenols actively aggregate and precipitate wine proteins at
286 room temperature,⁶³ most likely due to cross-linking protein
287 aggregates forming larger aggregates that are visible to the
288 naked eye. Cross-linking of proteins with nonprotein molecules
289 includes both covalent and noncovalent interactions. However,
290 because white wines are generally produced under nonoxidative
291 conditions, there are probably few cases of phenolic
292 compounds oxidizing to highly reactive quinones and
293 covalently cross-linking proteins. 294

Therefore, the growth in size of protein self-aggregates seems
295 also attributable to the cross-linking action of other matrix
296 components. This will be discussed further in the next section.
297 Elevated temperatures can increase polyphenol–protein
298 interactions and aggregation because increased temperatures
299 will increase the number of protein hydrophobic sites that are
300 exposed, as well as the intensity of the hydrophobic
301 interactions.⁶⁷ The exposure of hydrophobic sites also differs
302 in magnitude and consequence depending on protein type.⁶³
303 Polysaccharides can also influence haze formation, although
304 reports vary between stabilizing proteins against aggrega-
305 tion^{41,47,49} to inducing haze formation.⁵⁹ This variation may
306 be due to differences in the measured polysaccharide/protein
307

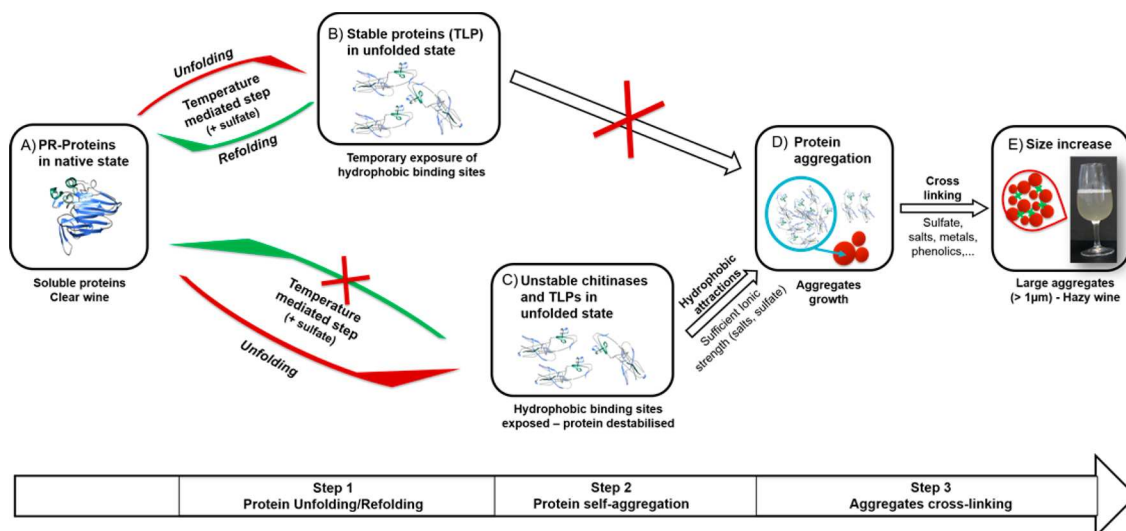


Figure 6. Revised unfolding and aggregation mechanisms of heat-unstable proteins in wine.

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

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

320 **Revised Mechanism of Protein Haze Formation.** Haze
 321 formation is caused by the unfolding and aggregation of grape-
 322 derived wine proteins and can lead to precipitation. The current
 323 model of wine protein aggregation indicates that protein
 324 unfolding and aggregation are separate events, as demonstrated
 325 through DLS experiments.¹³ Elucidating the mechanisms of
 326 haze formation has involved different analytical approaches
 327 characterizing naturally forming hazes from wine^{28,70} as well as
 328 heat-induced haze from real wines.^{14,71,72} Heat trials have
 329 indicated that proteins can unfold as wine is heated, although
 330 the wine becomes hazy only after cooling.¹³ The fractionation
 331 of heat-unstable wines into their component parts, such as
 332 proteins, polysaccharides, and phenolics, and the heat
 333 aggregation behavior studied via reconstitution experiments in
 334 model or real wines have also improved the knowledge of the
 335 haze-forming mechanism.^{28,53,54,61,63} Other significant advance-
 336 ments in understanding the mechanisms of wine haze include
 337 (i) the finding that sulfate plays an important role in
 338 hazing;^{54,61} (ii) the explanation of the role of nonproteinaceous
 339 wine components, particularly ionic strength,^{54,55} pH,^{58,66}
 340 organic acids,⁶⁵ and phenolic compounds;^{53,63,70} (iii) the
 341 development of an efficient protein purification method;¹⁷
 342 (iv) the release of the grape genome²⁶ and greater accessibility
 343 of proteomic techniques for protein characterization; (v) the
 344 discovery that TLPs and chitinases have different unfolding
 345 temperatures and unfolding/aggregation behavior;⁵⁷ and (vi)
 346 the solution of the crystal structure of thaumatin-like proteins.³⁰
 347 On the basis of these advancements, a new model of haze
 348 formation has been proposed (Figure 6).

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

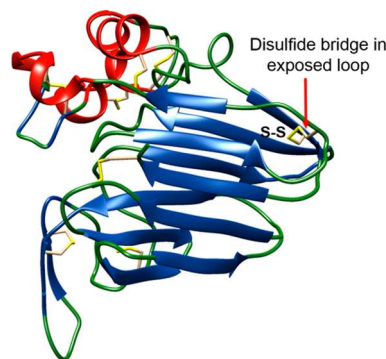


Figure 7. Backbone representation of the heat-unstable thaumatin-like protein 4JRU.³⁰ Disulfide bonds are yellow. The arrow indicates an exposed disulfide bond that could be susceptible to reduction by heat and sulfites.

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

372 case with TLPs. In the third and final stage of aggregation the
373 protein aggregates gradually become cross-linked due to the
374 actions of sulfates and polyphenols. Proteins form increasingly
375 larger aggregates until they reach a size that makes them visible
376 to the naked eye ($\geq 1 \mu\text{m}$) and eventually precipitate.^{13,54,55}
377 The presence of sulfate and salts can also neutralize protein net
378 charges and reduce the natural electrostatic repulsion between
379 similarly charged proteins, whereas polyphenols are more likely
380 to cross-link protein aggregates via hydrophobic interactions.
381 From this mechanism, the role of sulfate as a key wine matrix
382 component for the formation of haze becomes apparent. Sulfate
383 can potentially participate in each of the three stages of haze
384 formation by modifying the melt temperature of proteins in
385 stage 1, by screening the exposed protein charges in stage 2,
386 thus favoring aggregation of unfolded proteins,^{28,54} and by
387 mediating the aggregation of small protein aggregates probably
388 through a cross-linking action at stage 3 to modulate the final
389 haze formed.⁵⁴
390 Following the new insights into haze-forming mechanisms
391 and protein stability, the next stage of research will focus on
392 developing a new test for assessing the haze potential of white
393 wines. The conditions used in the current test, known as the
394 "heat test",⁷³ may overestimate the risk that a particular wine
395 will haze by denaturing both haze-forming and non-haze-
396 forming proteins. This can overpredict the amount of bentonite
397 needed to stabilize the wine, leading to less cost-effective
398 winemaking practices. Understanding the mechanisms of haze
399 formation and the structures of the proteins involved can also
400 lead to the development of new strategies for haze prevention
401 in white wines.

402 ■ PART II: BENTONITE ALTERNATIVES

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

413 Strategies 1 and 2 are most likely impractical from a wine
414 sensory quality perspective. Although it is possible to either
415 decrease the ionic strength of wine or remove polyphenols
416 using existing industrial-scale electrodialysis, ion exchange, and
417 fining technologies, these techniques would dramatically change
418 wine sensory attributes. Strategies 3 and 4 are potentially
419 related in practice, as the promotion or addition of specific
420 glycoproteins/proteoglycans/polysaccharides, including specific
421 yeast mannoproteins, could both stabilize wine proteins and
422 interfere with hydrophobic protein-protein interactions.⁵⁵
423 Protein stabilization with polysaccharides such as pectin and
424 carrageenan is well established in other beverages such as
425 milk⁷⁴ and beer, and the ability of a yeast mannoprotein to
426 stabilize wine proteins was attributed specifically to the glycan
427 portion of the proteoglycan.⁴⁹ However, in both cases, it is
428 unclear at what point protection against haze formation by
429 polysaccharides occurs: do the polysaccharides protect against
430 denaturation or, once proteins are denatured, protect against
431 aggregation? An unexplored alternative to polysaccharides that
432 would implement strategy 4 would be the addition of
433 surfactants to wines to prevent protein-protein interactions.

Surfactants such as polysorbates are commonly used in 434
processed foods and beverages to stabilize emulsions. However, 435
their use in wine is not currently permitted, they might not be 436
acceptable to consumers, and they could negatively affect foam 437
properties in sparkling wines. 438

The most promising strategies for developing bentonite 439
alternatives are strategy 5, the potential to degrade wine 440
proteins with enzymes, and strategy 6, the potential for 441
developing novel fining agents. The use of enzymes and novel 442
fining agents as bentonite alternatives will be discussed in more 443
detail in the following sections. 444

Protein Degradation Using Enzymes. Degrading haze- 445
forming proteins in wine with enzymes is a particularly 446
appealing alternative to bentonite because it minimizes wine 447
volume loss and aroma stripping. Ideally, effective enzymes 448
would be added to grape juice or ferment without the need for 449
later removal, such as with pectinases and glucanases.⁷⁵ The 450
degradation products of grape proteins may also be utilized by 451
yeast as nitrogen sources, potentially reducing the frequent 452
need for nitrogen additions (as diammonium phosphate) and 453
improving wine aroma quality.^{76,77} There are two types of 454
enzymatic activity relevant to wine protein degradation: the 455
hydrolysis of peptide bonds by proteases and the reduction of 456
disulfide bonds by protein disulfide reductases. Proteases 457
catalyze peptide bond hydrolysis through nucleophilic attack 458
induced either by an amino acid side chain of the protease, such 459
as for cysteine and serine proteases, or by an activated water 460
molecule, such as for metalloproteases and aspartic proteases.⁷⁸
461 Proteolytic enzymes are routinely used in the beverage industry,
462 for example, papain, a cysteine protease from papaya that is
463 used in beermaking,⁷⁹ and are therefore a viable option for use
464 in winemaking. Protein disulfide reductases could, theoretically,
465 destabilize and precipitate haze-forming proteins during
466 winemaking via the enzymatic reduction of disulfide bonds,
467 because the chemical reduction of disulfide bonds has been
468 shown to facilitate the unfolding of these proteins.⁶³ However,
469 there have been no published examples of protein disulfide
470 reductases being active under wine conditions. For this reason,
471 the search for wine-relevant enzymes to degrade haze-forming
472 proteins has focused on proteases and, at least since the 1950s,
473 researchers have tried to find proteases that destroy haze-
474 forming wine proteins under winemaking conditions.⁸⁰ The
475 difficulty in using proteases for specifically degrading haze-
476 forming proteins in wine is associated with the stability of the
477 proteins in wine-like conditions. Chitinases and TLPs are
478 characteristically highly resistant to proteases in their native
479 state due to their rigid peptide backbone structure^{72,81,82} and so
480 can tolerate the endogenous proteases that degrade many grape
481 proteins during crushing and pressing.²⁹ Grape TLPs, as with
482 PR proteins from other plant species, have been found to be
483 resistant to many different types of proteases and may even
484 inhibit the activity of some proteases.^{83,84} 485

Marangon et al.⁸⁵ developed a promising new protease 486
treatment that involves heating grape juice in the presence of a 487
heat-tolerant protease prior to fermentation to produce wine 488
that is free from haze-forming proteins. When juice is heated, 489
the proteins unfold and thus become susceptible to enzymatic 490
activity. The possible drawbacks of exposing grape juice or wine 491
to elevated temperatures are the requirements of high energy 492
inputs and the negative sensory implications,⁸⁶⁻⁸⁹ even though 493
it has been shown that when applied with care the potential 494
negative sensory changes can be contained, as well as the 495
energy requirements by optimizing the temperature and 496

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

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

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

539 Other potential sources of proteases that are active at wine
540 pH include endogenous winemaking sources such as grapes,
541 yeasts, and bacteria, because protein hydrolysis is known to
542 occur during winemaking.^{15,97,98} Endogenous grape (*V.*
543 *vinifera*) proteases including both cysteine and serine
544 proteases⁹⁹ have been found in berries and leaves,^{88,100,101}
545 although they are generally not well characterized.^{102–106} Grape
546 proteases are active at optima from pH 2 to 2.5 and from 60 to
547 70 °C,^{99,107} and the protease activity is generally short-lived
548 after pressing,^{88,100} with few exceptions.⁹⁹

549 Acid-tolerant yeasts and spoilage microbes have been found
550 to secrete proteases at wine pH, although the secreted protease
551 activity was not sufficient to stabilize wine.^{108–114} An
552 extracellular pepsin-like aspartic acid protease of 72 kDa was
553 characterized from a *Saccharomyces cerevisiae* isolate,¹¹⁵ one of
554 the few isolates that secrete protease activity.^{111,115} The
555 secreted yeast protease activity discovered by Younes et
556 al.^{115,116} was active at wine pH during grape juice fermentation,
557 although it did not affect grape PR proteins until after
558 fermentation when the wine was incubated at 38 °C for
559 prolonged periods. Nonetheless, the discovery of a secreted

protease from a *S. cerevisiae* isolate demonstrates that 560
proteolytic activity can occur prior to autolysis of the cell and 561
the release of a vacuolar acid protease, which is a previously 562
established mechanism of protease activity arising from yeast in 563
wine.¹¹⁷ The isolate that secretes protease activity is not 564
currently used commercially in winemaking,¹¹⁵ although it 565
could be used as a tool to develop new industrial wine yeast 566
strains that secrete protease. 567

Novel Fining Agents. Many novel fining agents and other 568
protein removal techniques with the potential to replace 569
bentonite have been explored in recent years. These include 570
seaweed polysaccharides, chitin, zirconium dioxide, and packed- 571
bed cation exchangers, as well as ultrafiltration techniques. 572
Novel fining agents must meet several criteria to effectively 573
compete with bentonite. They must be cost-effective and 574
nontoxic and must not degrade wine quality. 575

Negatively charged polysaccharides extracted from seaweeds 576
are a potential class of bentonite alternatives.¹¹⁸ Carrageenan is 577
a food grade polysaccharide that is extracted from red seaweed 578
and is currently used for protein stabilization in the beer 579
industry.¹¹⁸ It has been shown to be effective in stabilizing 580
white wines at low addition rates (125–250 mg/L), using only 581
one-third or less of the bentonite concentration required.¹¹⁹ 582
Carrageenan has been found to produce no deleterious sensory 583
impacts compared to bentonite-treated wines.¹²⁰ Although 584
those results are promising, residual amounts of carrageenan in 585
treated wines can potentially induce haze formation,^{118,119} and 586
this is likely to restrict commercial viability. Another potential 587
protein-adsorbing polysaccharide is chitin. Chitin is a 588
component of crustacean exoskeletons and, alongside its 589
derivatives, such as chitosan, is widely used in industrial 590
processes, including as a thickening agent for processed foods 591
and in pharmaceuticals.¹²¹ The structure of chitin also makes it 592
selective for binding to chitinases, and in-line systems 593
containing chitin can be effective in removing these particular 594
PR proteins from wine,¹²² even if its activity in stabilizing wine 595
has been recently challenged.¹²³ Chitin could potentially have 596
serious sensory impacts, however, by removing favorable wine 597
components such as positive aroma compounds.¹²⁴ 598

The ultimate objective of novel fining agents and protein- 599
adsorbing materials for the wine industry is to achieve wine 600
stabilization using in-line applications with minimum wine loss 601
and no extra processing steps. Zirconium dioxide (ZrO₂) is a 602
readily available protein-adsorbing material that can be 603
regenerated and thus reused.^{125,126} ZrO₂ has shown the 604
potential to remove haze-forming proteins when tested in 605
continuous and batch-wise application both during and post 606
fermentation.^{12,127,128} However, despite ZrO₂ showing promise 607
and the ability to be regenerated with a simple washing 608
procedure,¹²⁹ issues with the flow rates and high dosages 609
required limit its commercial viability. 610

Ultrafiltration can also be effective in removing haze-causing 611
proteins, although the process is not selective and can remove 612
other, desirable, wine components including polysaccharides.¹³⁰ 613
This application is not necessarily viable at winery-scale due the 614
expense of equipment. Packed-bed cation-exchangers could 615
improve process efficiency, and their effectiveness in removing 616
wine proteins has been demonstrated,¹³¹ but they have not as 617
yet been adopted for protein removal. 618

The mechanisms of wine haze formation have been revised 619
from the two-stage model to a three-stage model and now 620
include protein unfolding, protein aggregation, and the cross- 621
linking of aggregates to form a visible haze. Chitinases and 622

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

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