JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2007, 55, 1799–1807 1799

Sulfate—a Candidate for the Missing Essential Factor That Is Required for the Formation of Protein Haze in White Wine

KENNETH F. POCOCK,[†] GEOFFREY M. ALEXANDER,[§,#] YOJI HAYASAKA,[†] PATRIK R. JONES,[†,⊥,||] AND ELIZABETH J. WATERS*,[†,§]

The Australian Wine Research Institute, P.O. Box 197, Glen Osmond, South Australia 5064, Australia; School of Agriculture, Food and Wine, The University of Adelaide, Adelaide, South Australia 5005, Australia; and Cooperative Research Centre for Viticulture, P.O. Box 154, Glen Osmond, South Australia 5064, Australia

Protein haze formation in white wine is dependent on the presence of both wine protein and other unknown wine components, termed factor(s) X. The ability to reconstitute protein haze upon heating artificial model wine solutions (500 mg/L thaumatin, 12% ethanol, 4 g/L tartaric acid) to which candidate components were added was employed to identify factor(s) X. No protein haze was formed in the absence of additives. The individual or combined addition of caffeic acid, caftaric acid, epicatechin, epigallocatechin-*O*-gallate, gallic acid, or ferulic acid at typical white wine concentrations did not generate protein haze. However, PVPP fining of commercial wines resulted in a reduction in protein haze, suggesting that phenolic compounds may play a modulating role in haze formation. To elucidate the nature of the unknown factor(s) wine was fractionated and fractions were back-added to model wine and tested for their essentiality. Wine fractions were generated by ultrafiltration, reverse-phase chromatography, and mixed-mode anion-exchange and reverse-phase chromatography. The only purified fraction containing the essential component(s) was free of phenolic compounds, and analysis by mass spectrometry identified sulfate anion as the dominant component. Reconstitution with $KHSO_4$ using either commercially available thaumatin or wine proteins confirmed the role of sulfate in wine protein haze formation. The two main wine proteins, thaumatin-like protein and chitinase, differed in their haze response in model wines containing sulfate. Other common wine anions, acetate, chloride, citrate, phosphate, and tartrate, and wine cations, $Fe^{2+/3+}$ and $Cu^{+/2+}$, when added at typical white wine concentrations were not found to be essential for protein haze formation.

KEYWORDS: White wine; protein haze; turbidity; sulfate; wine anions; phenolics; thaumatin; chitinase

INTRODUCTION

Protein hazes can form in white wines after bottling (*1*). The wine proteins responsible for the haze are pathogenesis-related proteins from the grape that survive the vinification process and remain in wine unless removed by downstream winery operations (*2*). Although these proteins are the major constituent of haze, several papers in the literature suggest that other wine components are involved in wine protein haze formation.

The phenolic compounds are obvious candidates as it is well-established that they are involved in protein hazes in beer and fruit juices (*3*). The interaction of grape protein with tannin was suggested more than 40 years ago (*4*) and, in 1973, Somers and Ziemelis (*5*) proposed that up to 50% of white wine protein was bound to flavonoid material. They used this information to explain the variations noted by others (*6*) in protein stability among wines with similar total protein concentrations, and they speculated that protein haze is due to the fractions of residual wine proteins which have been rendered prone to precipitation by interaction with phenolics. Yokotsuka and colleagues (*7*) found that tannins isolated from wines interacted with isolated must proteins to form a haze and that proteins isolated from grape must did not produce a visible haze in the presence of non-tannin phenolics from wine. These studies were not, however, undertaken under conditions identical to those commonly encountered in commercial white wines. Procyanidins (tannins, 0.02−4.9% w/w) have been detected in recovered wine protein haze (*8*).

Metal ions such as copper and iron have been implicated in the formation of protein hazes in white wines, but as they are also associated with hazes that do not contain protein, their role in protein haze formation is very poorly understood. Copper

* Corresponding author (fax +61 8 8303 6601; e-mail Elizabeth.Waters@awri.com.au).
[†] The Australian Wine Research Institute.
[§] The University of Adelaide.
[#] Present address: Brown Brothers Milawa Vineyard Pty. Ltd., P.O. Box 44, Milawa, VIC 3678, Australia.
[⊥] Cooperative Research Centre for Viticulture.
[||] Present address: Fujirebio Inc., 51 Komiya-cho, Hachioji-shi, Tokyo 192-0031, Japan.

10.1021/jf062658n CCC: $37.00 © 2007 American Chemical Society
Published on Web 02/13/2007

Table 1. Composition of Wine Protein Fractions A and I

fraction	protein content[a] (% w/w)	M_r of major protein component[b]	protein type[c] (%)
A	98	21269	major thaumatin-like protein, 100
I	100	25474	chitinase, 94
			thaumatin-like proteins, 6

[a] Determined from the sum of the masses of the amino acids present after acid hydrolysis of the protein fraction. [b] Determined by electrospray mass spectrometry. [c] By reverse-phase HPLC (*19*) and expressed as a percentage of the total protein detected and quantified as cytochrome *c* equivalents.

concentrations in wine were found to decrease after protein haze removal (*9*), suggesting copper was part of the protein precipitate.

The effect of wine polysaccharides on protein haze has also been documented. Fifteen different polysaccharides from diverse sources were added to wines before protein hazes were induced, and they either did not affect or increased protein haze levels (*10*). Another study showed that polysaccharides increased protein instability, particularly at moderate to high temperatures (*11*). However, the level of polysaccharide in both studies was much greater than that reported (*12*) in wines. A multifactorial study (*13*) showed a particular polysaccharide (pectin) to be important in haze formation. However, because pectolytic enzymes are commonly used in white winemaking and ethanol precipitates pectins, the levels of pectins in commercial white wines are very low.

The present literature strongly suggests that an as yet unknown non-proteinaceous wine component (factor X) is required for visible protein haze to be formed in commercial white wine. Nevertheless, clear cause-and-effect relationships remain to be demonstrated to explain why wine proteins alone are not sufficient to render model wine prone to haze formation. It must also be noted that some wine components may have a negative effect on protein haze formation, and yeast-derived mannoproteins are well documented to play such a role in white wine (*14*−*16*).

We describe here experiments to confirm that factor X exists in commercial wines and is required for protein hazes to form. Furthermore, a search for wine components that act as factor X within the concentration range commonly found in commercial wines revealed a compound hitherto unknown to play such a role. These data allow us to propose a mechanism for protein haze formation in white wine that differs from that in other beverages.

MATERIALS AND METHODS

Materials. Epicatechin, epigallocatechin-*O*-gallate, ferulic acid, gallic acid, polyvinylpolypyrrolidone (PVPP), and thaumatin were purchased from Sigma-Aldrich (Sydney, Australia). Caftaric acid was from Dalton Chemical Laboratories (Toronto, Canada) and caffeic acid from Fluka AG (Buchs, Switzerland). Grape seed tannin with a degree of polymerization (dp) of 3 was isolated from Shiraz grape seed as described (*17*).

Thaumatin-like proteins and chitinases were purified from a 1993 Muscat Gordo Blanco wine as previously described (*18*). Briefly, crude wine proteins were precipitated by adding ammonium sulfate to saturation (approximately 500 mg/L), and the precipitate was resuspended in 30 mM citric acid (pH 3.5) and desalted by ultrafiltration (10 kDa cutoff). The crude wine protein fraction was fractionated by anion-exchange chromatography at pH 8.0 on Fast Flow Q Sepharose with a NaCl gradient. Thaumatin-like protein (fraction A) eluted between 60 and 75 mM NaCl and chitinases (fraction I) between 150 and 160 mM NaCl. Fractions were collected, lyophilized, and stored at −20 °C. The protein compositions of fractions A and I were confirmed by reverse-phase HPLC (*19*) and mass spectroscopy (*20*).

Table 2. Details of Wines Used

year, variety	source	pH	alcohol (% v/v)	protein[a] (mg/L)
2001, Sauvignon Blanc	Yarra Valley, VIC	3.07	12.5	nd[b]
2004, Chardonnay (unwooded)[c]	Langhorne Creek, SA	3.42	11.9	134
2004, Chardonnay (wooded)[c]	Langhorne Creek, SA	3.36	11.9	130
2004, Gordo	Mildura, VIC	3.47	12.5	255
2004, Riesling	Eden Valley, SA	2.97	12.1	150
2004, Sauvignon Blanc	Riverland, SA	3.34	10.5	211
2004, Semillon	Hunter Valley, NSW	3.29	9.9	87
2004, Semillon	Adelaide Hills, SA	3.22	11.0	223
2004, Sultana	Mildura, VIC	3.43	12.1	28

[a] Sum of thaumatin-like and chitinase proteins determined by reverse-phase HPLC (*19*) and expressed as cytochrome *c* equivalents. [b] Not determined; assumed to be negligible as wine was overfined with bentonite. [c] Both wines were made from the same lot of juice; unwooded was held in a stainless steel tank, and wooded was held in a stainless steel tank containing oak planks.

Wines and Model Wines. A commercial 2001 Sauvignon Blanc wine, which had been supplied clarified but not fined with bentonite, was fined with a sodium bentonite at 2 g/L as described (*21*) to remove protein and was filtered through a 0.45 μm membrane. Details of this wine are given in **Table 2**.

Eight commercial 2004 dry white wines, which had been clarified but not fined with bentonite, were supplied by two wine companies. Details of the wines are given in **Table 2** and in ref *21*.

A commercially bottled 2003 Chardonnay wine from McLaren Vale, South Australia, was purchased from a local retail outlet.

Model wines were prepared containing 12 or 24% by volume of ethanol in water, to which 4 or 8 g/L tartaric acid, respectively, was added. Sodium hydroxide solution was used to adjust the pH. When model wine with 24% v/v ethanol was used, the final ethanol concentration was adjusted to 12% v/v after additions were made.

Analytical Methods. The alcohol content of wines was measured by near-infrared spectroscopy (InfraAlyzer 260, Bran + Luebbe, Madison, WI) as per the manufacturer's instructions. Sulfur dioxide and the pH of wines and model wine samples were analyzed by the aspiration method and the standard pH procedure, respectively (*22*). Proteins in wines and in samples containing thaumatin and purified wine protein fractions were analyzed by HPLC (*19*). Analysis of the phenolic profile of the wine fractions was performed using C18 HPLC as described (*23*). Total phenolics of wines were estimated by a spectrophotometric method (*24*).

Haze Formation Procedure. The protein stability test (*25*) was used to induce haze in triplicate samples as follows. An aqueous solution of thaumatin was added at 500 mg/L to 3 mL aliquots of the overfined 2001 Sauvignon Blanc wine and to model wine. The absorbances of these samples, together with a control wine and model wine with no added thaumatin, were measured at 540 nm in 1 cm cuvettes. These samples were then put into Kimble tubes (103 mm × 14 mm diameter), capped, and heated for 6 h at 80 °C in a heating module fitted with three heating blocks, each containing nine holes of 17 mm diameter (Pearce ReactiTherm III, Laboratory Supply Pty. Ltd., Edwardstown, SA, Australia). After heating, the tubes were cooled in an ice bath and then held at 4 °C overnight. The presence of hazes after heat treatment was determined visually and estimated by measuring absorbance at 540 nm.

Effect of Phenolics. Phenolic compounds (caffeic acid, caftaric acid, epicatechin, epigallocatechin-*O*-gallate, gallic acid, ferulic acid, and dp3 grape seed tannin) were added to model wine of 24% v/v ethanol at pH 3.3, and samples were diluted to a final volume of 1 mL, containing 12% (v/v) ethanol, 500 mg/L thaumatin, and the phenolic compounds at either 10, 20, 25, 50, or 100 mg/L in 2 mL capped plastic tubes (Trace Plastics, Melbourne, Australia). Hazes were induced by

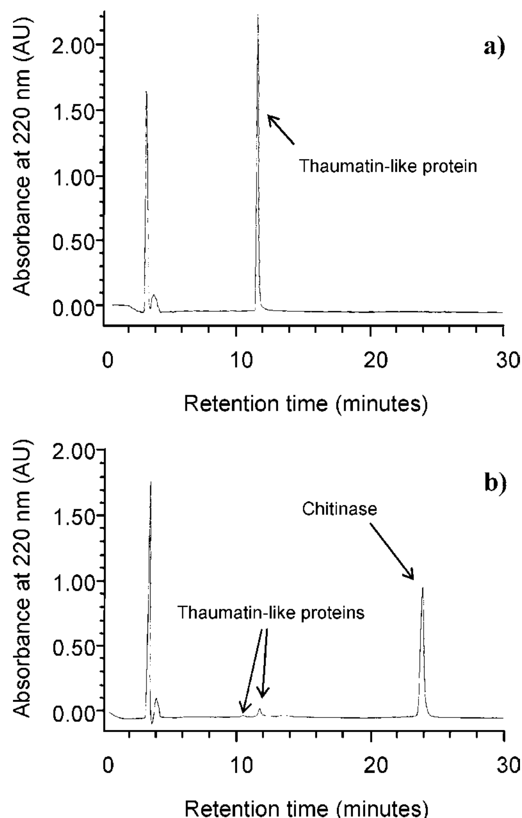

Figure 1. Composition of purified wine protein fractions: (**a**) fraction A, the major thaumatin-like protein; (**b**) fraction I, chitinase by reverse-phase HPLC separation.

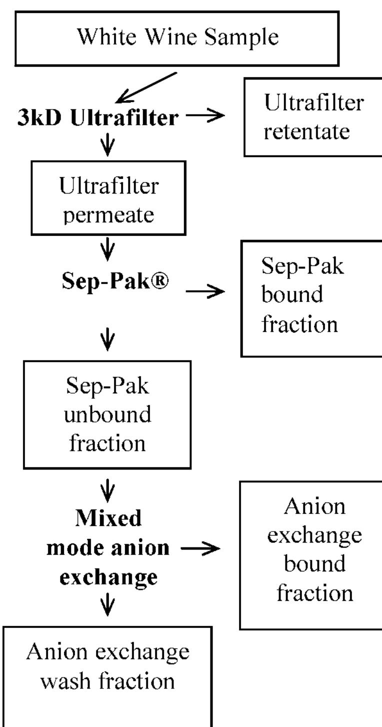

Figure 2. Fractionation scheme.

heating as described above. The absorbances of the samples were determined after heating and after centrifugation (10000*g*, 3 min) to remove the haze. The final after-heating value was determined as the difference between the measurements before and after centrifuging, to take into account color changes caused by heating. The model wine containing 500 mg/L thaumatin was used as a negative control, and the 2003 commercial Chardonnay wine containing 500 mg/L thaumatin was used as a positive control.

Effect of Overfining Wines with PVPP. PVPP was added at 10 and 100 g/L to 10 mL samples of the eight 2004 unfined wines (Chardonnay, unwooded; Chardonnay, wooded; Muscat Gordo; Riesling; Sauvignon Blanc; Semillon Hunter Valley; Semillon Adelaide Hills; Sultana) to massively overfine them and remove most or all phenolic compounds (*24*). After 90 min on a rotating wheel mixer at room temperature, the samples were centrifuged (2500*g*, 10 min, 20 °C). The supernatants were then filtered through 0.45 μm membranes. Hazes were induced in untreated controls and PVPP-treated samples as described above. Total phenolic content was determined on unheated samples by spectrophotometry (*24*).

Effect of Metals. Freshly made stock solutions of ferrous sulfate and cuprous chloride in Milli-Q water were sparged with nitrogen. These were added to give, separately, ferrous ion concentrations of 2 and 10 mg/L and cuprous ion concentrations of 1 and 5 mg/L to model wine containing thaumatin. The additions were made using an anaerobic hood to prevent oxidation.

Freshly made stock solutions of ferric sulfate and cupric chloride in Milli-Q water were added to give separately ferric ion concentrations of 2, 4, 6, 8, and 10 mg/L and cupric ion concentrations of 1 and 5 mg/L to model wine containing thaumatin. Hazes were induced in the samples as described above.

Effect of Sulfate. Potassium hydrogen sulfate solution was added to model wine to give sulfate contents of 0, 0.5, 1.0, 1.5, and 2.0 g/L, and 500 mg/L thaumatin was added to all samples. Sample absorbances at 520 nm in a 1 cm cuvette were measured before and after heating at 80 °C for 6 h.

Effect of Anions, Using Wine Proteins. Purified wine protein fractions (**Table 1**) were added separately, and as 1:1 mixtures, at 150 mg/L to model wine, with and without additions of anions commonly found in wine, at concentrations within the range of concentrations reported in wine and juice (**Table 5**). In addition, samples containing a mixture of anions, simulating grape juice composition (*26*), were tried (**Table 5**). These samples were made up to a total volume of 1 mL, in 2 mL plastic capped tubes, the pH was adjusted to 3.0, and hazes were induced as described above. Protein contents before and after heating were also measured.

Separation of Fractions Containing the Essential Factor from Wine. A summary of the fractionation scheme is given in **Figure 2**. A commercially bottled Chardonnay wine (100 mL) was lyophilized, and the residue was dissolved in 20 mL of Milli-Q water. Three lots of this solution (each 4 mL) were treated using three 3 kDa ultrafilters (Amicon YM-3, Bedford, MA) as per the manufacturer's instructions. The filtrates (ultrafilter permeates) were collected, and each ultrafilter retentate was washed with at least 10 volumes of Milli-Q water. The pooled retentate was made up to a final volume of 12 mL with Milli-Q water. A 7 mL aliquot of ultrafilter permeate was loaded onto a tC18 Sep-Pak cartridge (Waters Corp., Milford, MA), previously equilibrated with 5 mL of Milli-Q water, 5 mL of methanol, and 20 mL of Milli-Q water. The Sep-Pak cartridge flowthrough (Sep-Pak unbound fraction) was collected and the cartridge subsequently washed with 5 mL of Milli-Q water. To collect the Sep-Pak cartridge retentate (Sep-Pak bound fraction), two 5 mL aliquots of methanol were added to the cartridge. All recovered fractions were evaporated to dryness under reduced pressure at 40 °C and subsequently adjusted to 7 mL with Milli-Q water.

The Sep-Pak unbound fraction was further fractionated. A 1 mL aliquot of the Sep-Pak unbound fraction was loaded onto a MAX mixed-mode anion-exchange cartridge (Waters Corp.), previously equilibrated as described above for the tC18 Sep-Pak cartridge. The pH of the Sep-Pak unbound fraction was not adjusted and was likely to be close to the pH of the wine (approximately 3.4). The column was sequentially washed with 5 mL of 2% ammonium hydroxide in water, and this fraction was pooled with flowthrough material from the initial loading (anion-exchange wash fraction), 5 mL of methanol, and 5 mL of 2% formic acid in methanol (anion-exchange bound fraction). The fractions were evaporated to dryness under reduced pressure at 40 °C and made up to 1 mL with Milli-Q water.

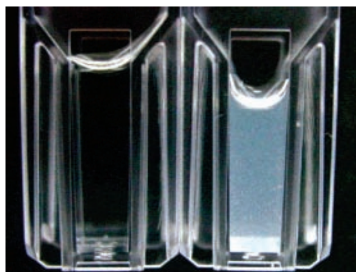

Figure 3. Haze produced by heating thaumatin in either model wine (left) or a commercial bentonite fined white wine (right).

To assess any contribution to haze by the cartridges or solvents, these procedures were repeated using Milli-Q water as the sample.

The fractions were reconstituted at approximately the original wine concentration in model wine at pH 3.3, and 500 mg/L thaumatin was added before haze was induced as described above for phenolics. The absorbances of the samples were determined after heating and after centrifugation (10000*g*, 3 min) to remove the haze. The final after-heating value was determined as the difference between the measurements before and after centrifuging, to take into account the color changes caused by heating. The model wine with thaumatin was used as a negative control, and the original wine with thaumatin was used as a positive control.

The phenolic composition of each of the purified wine fractions was checked by HPLC analysis (see above).

Identification of an Essential Factor by Mass Spectroscopy. Sultana wine (390 mL) was ultrafiltered through a 3 kDa cutoff membrane using a pressurized 500 mL stirred cell under nitrogen. Total volume of retentate was 33 mL, and 7 mL of the ultrafiltrate was fractionated as described above and in **Figure 2**. The anion-exchange wash fraction was freeze-dried and was tested to ensure that it did not form a haze in model wine but did form a haze in model wine with added thaumatin, as described above. A second sample of anion-exchange wash fractions was submitted for mass spectrometric analysis as 2% ammonium hydroxide solutions. A 2% ammonium hydroxide solution was used as a control. Mass spectrometric analysis was performed on an API-300 triple-quadrupole mass spectrometer combined with an electrospray ion source (Applied Biosystems/MDS Sciex, Foster City, CA). A sample solution was infused into the electrospray ion source at a flow rate of 5 μL/min with a syringe pump (Cole-Parmer, Vernon, IL). Negative ion mass spectra from m/z 30 to 1000 were recorded with a step size of 0.1 amu and a scan time of 3 s. The electrospray needle, orifice, and ring potentials were set at −4500, −30, and −250 V, respectively. The curtain (nitrogen) and nebulizer (air) gases were set at 8 and 12 (arbitrary units), respectively. For the tandem mass spectrometric (MS/MS) experiment, nitrogen gas was used as the collision gas setting at 2 (arbitrary unit), and the collision energy was set at 30 V. Mass spectrometric data acquisition and analysis were carried out with Analyst 1.4.2 software (Applied Biosystems/MDS Sciex).

RESULTS AND DISCUSSION

Haze Formation in Model Wine Compared to Wine. Commercially available thaumatin derived from *Thaumatococcus daniellii* was used as a test protein in the initial stages of this work because of its similarity to thaumatin-like (TL) wine proteins (*2*, *27*). When added to a protein-stable commercial wine (2003 Chardonnay), it formed a heavy haze upon heating (**Figure 3**; **Table 3**), but there were no hazes formed after heating when thaumatin was added to a model wine (an aqueous solution of ethanol and tartaric acid at pH 3.0; **Figure 3**), in a model wine control (**Table 3**), or in the commercial wine without protein addition (data not shown). These results suggest that wines contain one or more factors necessary for protein haze formation that are not present in model wine. The above method formed the basis for an assay in which candidate components were tested by addition to model wine containing thaumatin and subjected to a heat test. To determine the identity of essential protein haze factors, a range of commercially available candidate compounds were tested at levels at and above those commonly encountered in commercial wines.

Table 3. Impact of Addition of Various Wine Components to Model Wine Containing Thaumatin (500 mg/L) on Haze Produced after Heating

component or fraction added	haze (ΔA_{540nm}) after heating[a] mean	SD
negative control (no addition)	−0.005 to 0.014[b]	0.005[b]
caftaric acid (20 mg/L)	0.010	0.030
caftaric acid (100 mg/L)	0.005	0.014
epicatechin (20 mg/L)	−0.003	0.005
epicatechin (100 mg/L)	0.003	0.006
epigallocatechin-*O*-gallate (20 mg/L)	0.009	0.001
epigallocatechin-*O*-gallate (100 mg/L)	0.015	0.006
caffeic acid (20 mg/L)	0.010	0.001
caffeic acid (100 mg/L)	0.013	0.004
gallic acid (20 mg/L)	0.008	0.001
gallic acid (100 mg/L)	0.014	0.006
mixture of the five phenolics above (20 mg/L each)	0.013	0.005
ferulic acid (10 mg/L)	−0.003	0.002
ferulic acid (100 mg/L)	−0.003	0.001
grape seed tannin (dp3, 25 mg/L)	0.008	0.000
grape seed tannin (dp3, 50 mg/L)	0.018	0.002
Fe^{2+} (2 mg/L) added as ferrous sulfate	0.002	0.004
Fe^{2+} (10 mg/L) added as ferrous sulfate	0.243	0.001
Fe^{3+} (2 mg/L) added as ferric sulfate	0.001	0.007
Fe^{3+} (10 mg/L) added as ferric sulfate	0.369	0.032
Cu^{+} (1 mg/L) added as cuprous chloride	−0.001	0.003
Cu^{+} (5 mg/L) added as cuprous chloride	0.005	0.012
Cu^{2+} (1 mg/L) added as copper sulfate	−0.001	0.003
Cu^{2+} (5 mg/L) added as copper sulfate	−0.043	0.062
ultrafilter permeate	1.066	0.068
Sep-Pak unbound fraction	0.661	0.041
Sep-Pak bound fraction	0.000	0.008
anion-exchange wash fraction	1.470	0.052
anion-exchange bound fraction	0.003	0.002
sulfate (0.5 g/L)	0.021	0.005
sulfate (1.0 g/L)	0.113	0.012
sulfate (1.5 g/L)	0.296	0.005
sulfate (2.0 g/L)	0.405	0.041

[a] Values are from at least three replicates. Positive control for all experiments was commercial white wine with thaumatin (500 mg/L) added. Haze values ranged from 1.4 to 1.5. [b] Range from all experiments conducted.

Effect of Phenolics on Haze Formation. A range of phenolic compounds found in white wines was added to model wine containing thaumatin. No hazes formed after heating in any of the samples containing monomeric phenolic compounds and trimeric procyanidins added at normal wine concentrations and at 5 or 10 times normal concentrations (**Table 3**). A mixture of five of these compounds, each at normal wine concentrations, also failed to promote the formation of haze (**Table 3**). The addition of trimeric procyanidins at concentrations up to 50 mg/L also failed to produce protein haze. White wines generally contain far less than 50 mg/L procyanidins (*28*, *29*). These results show that the phenolic compounds tested here are not essential factors. This does not exclude the possibility that other untested monomeric or polymeric phenolic compounds, or mixtures of these, constitute essential factors.

Wines were also fined at two levels with PVPP, and the resultant estimated concentration of phenolic compounds and the level of haze produced after heating are shown in **Table 4**. The protein content of the wines after treatment was not determined but was not expected to substantially change. At

Table 4. Effect of Overfining Wines with PVPP on Haze Formation after a Heat Test and on Total Phenolics Concentration

	PVPP fining rate					
	0 g/L		10 g/L		100 g/L	
wine	haze[a] (ΔA_{540nm})	total phenolics[b] (mg/L)	haze[a] (ΔA_{540nm})	total phenolics[b] (mg/L)	haze[a] (ΔA_{540nm})	total phenolics[b] (mg/L)
Chardonnay (unwooded)	0.21	184	0.12	85	0.085	21
Chardonnay (wooded)	0.20	208	0.11	107	0.075	38
Muscat Gordo	0.53	197	0.48	104	0.42	43
Riesling	0.15	193	0.086	95	0.05	29
Sauvignon Blanc	0.21	83	0.19	18	0.16	–16[c]
Semillon Hunter Valley	0.075	95	0.043	60	0.016	22
Semillon Adelaide Hills	0.14	104	0.083	34	0.069	–8[c]
Sultana	0.042	94	0.020	31	0.019	–15[c]

[a] Difference between heated and unheated. [b] Caffeic acid equivalents + catechin equivalents. [c] Negative values may result from estimating concentration by applying the equation developed by Somers and Ziemelis (*24*) to the raw data. This equation uses a correction factor, based on the data from many wines, to account for absorbance due to nonphenolic material.

the 1% (10 g/L, w/v) and 10% (100 g/L, w/v) fining rates, total phenolic concentrations were reduced substantially. All PVPP fined wines formed less haze than the unfined controls after heating. In two wines with the initial lowest protein contents (Semillon Hunter Valley and Sultana), the differences in absorbance values for the samples fined with PVPP at 100 g/L were <0.02, which has been used as a pass−fail point in protein stability tests in the past (*21*).

The PVPP fining experiment confirms previous results (*5*, *7*, *8*) which suggest that phenolic compounds are involved in protein haze formation, although it is possible that wine proteins and other unknown wine components were also affected by the PVPP treatments. If the effect of PVPP treatment on haze formation mainly resulted from the removal of phenolic compounds, it would be of great interest to further elucidate what fraction or class they belong to.

Effect of Metals on Haze Formation in Model Wine. Iron or copper ions were added to the model wine under anaerobic conditions at each of their oxidation states and at levels similar to, and significantly above, those found in wine. Both metal ions might be expected to be in the free, unbound state after addition because little material exists in model wine to bind them. However, the degree of free versus bound ions cannot be predicted from the model wine composition and would need to be assessed to produce a clear picture. Fe and Cu, irrespective of the form or association, have been estimated to be present in commercial wines at concentrations of up to 5 and 2.5 mg/L, respectively (*30*).

A heavy haze formed after heating in the 10 mg/L ferrous or ferric iron samples (**Table 3**). Heavy hazes formed in the samples containing 6 and 8 mg/L iron in the ferric form, also (data not shown). No hazes formed in any of the other samples, including all of those in which wine relevant concentrations of the ions were present. These data suggest that iron and copper are not essential factors under normal wine conditions. As no firm candidates for essential factors were obtained by addition of commercial compounds at concentrations commonly found in commercial wines, a search for essential factors by purification was initiated.

Separation of Fractions Containing Factor X from Wine. A white wine was fractionated by ultrafiltration followed by tC18 solid-phase extraction, then by mixed-mode reverse-phase/anion-exchange solid-phase extraction (**Figure 2**), and the resulting fractions were added to model wine samples containing thaumatin. The formation of hazes upon heating indicated which fractions contained potential essential factors. Hazes formed only in the presence of the ultrafilter permeate, the unbound fraction from the tC18 Sep-Pak, and the wash fraction from the mixed-mode anion-exchange resin (**Table 3**). The presence of the essential factor in the ultrafilter permeate suggested that it had a molecular mass of <3 kDa, in the Sep-Pak unbound fraction that it is hydrophilic, and in the anion-exchange wash fraction that it is not both hydrophobic and negatively charged (see http://www.waters.com/WatersDivision/pdfs/lcSP.pdf, page 10, for a discussion of the mode of action of this cartridge). HPLC analysis showed that most of the phenolics present in the original wine were still present in ultrafilter permeate (data not shown). Reduced amounts of monomeric phenolics and no polymeric phenolics were detected in the tC18 Sep-Pak unbound fraction, and only trace amounts of monomeric phenolics were present in the mixed-mode anion-exchange wash fraction (data not shown). These results, together with those above, indicate that factor X in the studied wine was not a phenolic compound.

Identification of Factor X by Mass Spectroscopy. A mixed-mode anion-exchange fraction was isolated from Sultana wine using the same procedure as above and subjected to mass spectrometric analysis as a 2% ammonium hydroxide solution. A 2% ammonium hydroxide solution, recovered from a Max cartridge without loading any wine fraction, was used as a control. Panels **a** and **b** of **Figure 4** show the mass spectra of the control and anion-exchange wash fraction, respectively. In comparison to the control, the anion-exchange wash fraction gave the more complicated mass spectrum featuring the dominant ion at m/z 97 with the additional ions at m/z 87, 115, 133, and 149, which were not observed in the control. These ions were considered to be derived from wine constituents that might be candidates for essential factors. The ion m/z 97 was also detected in the control, but its intensity was considerably lower than that in the anion-exchange wash fraction, suggesting that the anion-exchange wash fraction contained a wine constituent greatly deriving m/z 97. To identify a compound responsible for the production of m/z 97, the control and the anion-exchange wash fraction were analyzed by the MS/MS technique. The product ion spectrum of m/z 97 detected in the anion-exchange wash fraction showed only one fragment ion at m/z 80 and was obviously different from that in the 2% ammonium hydroxide control having m/z 79, 61, and 35 as fragment ions (**Figure 5a**,**b**). It was evident that the majority of the ion composition of m/z 97 was different between the samples. The product ion spectrum of the anion-exchange wash fraction appeared to be consistent with HSO_4^- (m/z 97) and SO_3^- (m/z 80) ions and was identical to that of potassium

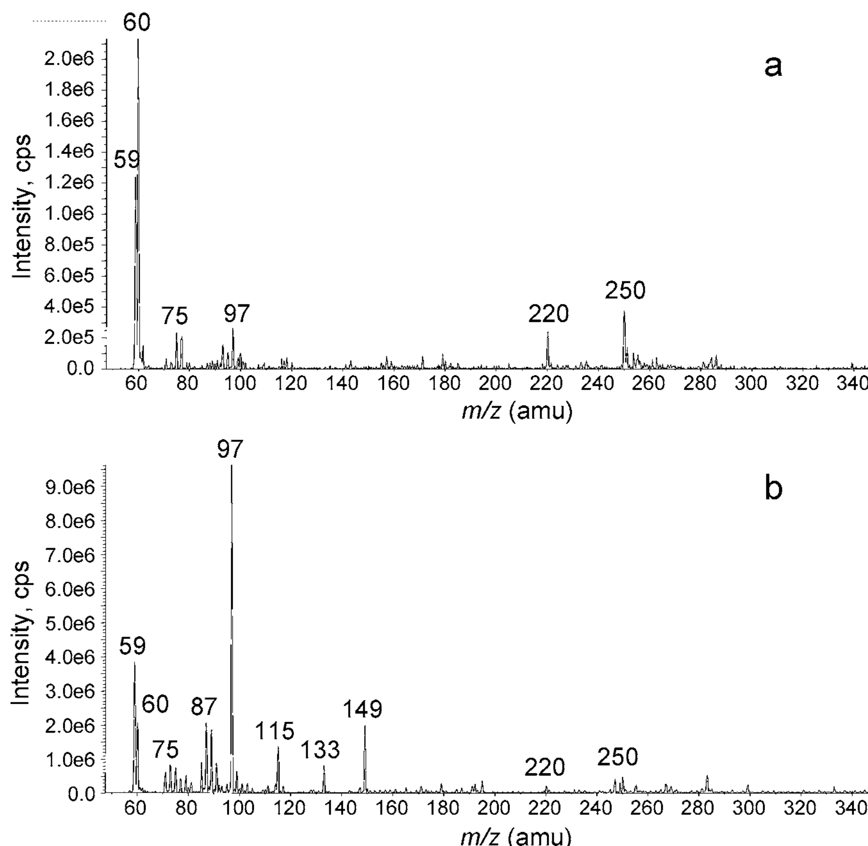

Figure 4. Negative ion mass spectra of (**a**) 2% ammonium hydroxide and (**b**) anion exchange wash fraction in 2% ammonium hydroxide.

hydrogen sulfate ($KHSO_4$) (**Figure 5c**). Thus, the most intense ion detected in the anion-exchange wash fraction was identified as sulfate ion. Furthermore, sulfate (*m/z* 97) can be differentiated from phosphate ($H_2PO_4^-$: *m/z* 97), which is fragmented to *m/z* 79 (PO_3^-) instead of *m/z* 80 (SO_3^-).

Confirmation That Sulfate Is Required for Haze Formation. When sulfate at 1 g/L and higher was added (as HSO_4^-) to model wine containing thaumatin, a haze developed after heating (**Table 3**). It is of interest to note that none of the sulfate additions to the model wine containing thaumatin were able to generate as much haze as the fractions isolated from wine, including the anion-exchange wash fraction. It is possible that the fractions may have been enriched with sulfate to levels above those tested or that other wine components present in these fractions could have amplified the effects of the sulfate also present.

To confirm the role of sulfate with authentic wine proteins, a range of sulfate additions (as SO_4^{2-}) were also added to model wine containing purified wine proteins (**Figure 6**). The results suggest that the thaumatin-like protein (150 mg/L) required approximately 150 mg/L sulfate, and the chitinase (150 mg/L) required approximately 15 mg/L sulfate, for visible haze formation in the model wine system, defined as an increase after heating of absorbance at 520 nm of >0.02 (*21*). When both wine protein fractions were present together in a model wine, each at 75 mg/L, no haze formed with 100 mg/L sulfate, but haze occurred when 400 mg/L sulfate was present (other sulfate concentrations not assessed, data not shown). Controls without wine protein fractions did not form hazes.

These data strongly suggest that sulfate is required for protein haze formation in white wines. Sulfate is always present in juice and wine; generally at concentrations that were effective in this study. Adrian Coulter and Mark Gishen (2005, personal communication) collated results from sulfate analyses of 895 wines, covering 20 varieties including their blends, submitted to the Analytical Service of the Australian Wine Research Institute between 1994 and 1997. The range of sulfate was from 56 to 1780 mg/L, with a mean of 385 mg/L. Sulfate concentrations in Australian grape juices of up to 850 mg/L, with a mean of 185 mg/L, have been reported (*31*).

Role of Other Wine Anions in Haze Formation in Model Wine. Other anions present in wines were tested for their ability to act as factor X. Details of the levels tested and the ranges encountered in wines are given in **Table 5**. No hazes occurred after heat tests in samples containing wine proteins (thaumatin-like proteins or chitinases) together with acetate, chloride, citrate, or phosphate (data not shown). Control samples that contained these anions separately but no wine proteins did not form hazes after heating. Therefore, these anions are not essential factors for protein haze formation in model wine. Hazes formed when a mixture of these four anions, together with malate and sulfate, were heated with either wine protein fraction. Control samples without wine proteins did not form hazes. Thus, sulfate is an essential factor for protein haze formation in model wines containing wine proteins, and the other anions tested do not inhibit the formation of protein haze in model wine when sulfate is present under the conditions of this study.

General Discussion. The grape proteins involved in haze formation in wines probably exist in young wines as freely soluble globular entities with a net positive charge. We hypothesize that the first step in protein haze formation in wines is protein denaturation, a process accelerated by heating. The denatured proteins aggregate into large enough protein particles to be visually detected as haze, a process that may be affected either positively or negatively by non-proteinaceous wine components.

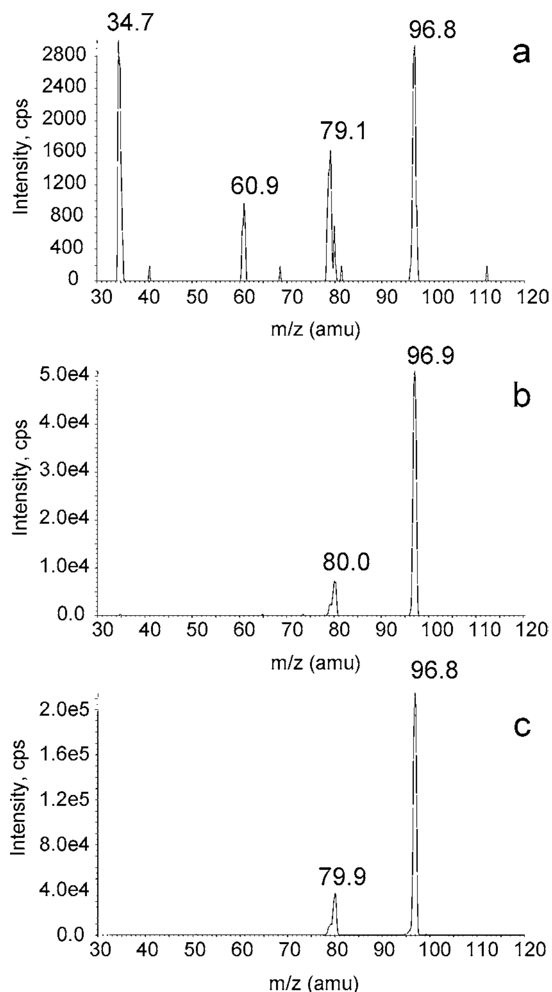

Figure 5. Product ion spectra of m/z 97 detected in (**a**) 2% ammonium hydroxide control, (**b**) anion-exchange wash fraction in 2% ammonium hydroxide, and (**c**) $KHSO_4$.

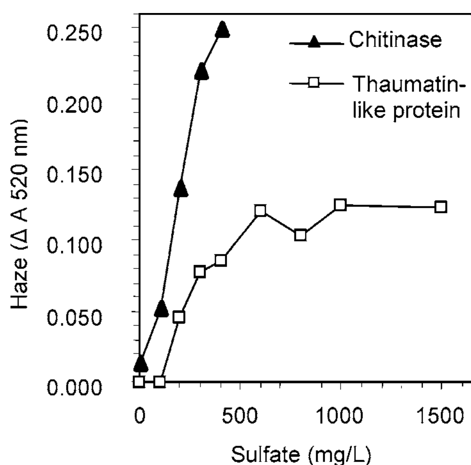

Figure 6. Effect of increasing sulfate concentration, added as potassium hydrogen sulfate, on the haze produced by heating wine proteins (150 mg/L) in model wine.

Sulfate is one of the Hofmeister series of anions, a ranking of the ability of various ions to precipitate proteins (*32*). In simple terms, precipitation of proteins by kosmotropic anions occurs due to "salting out"—a competition between the anion and the protein for water of solvation resulting in a loss of water from the protein surface. This process is classically applied in ammonium sulfate precipitation as the first step in many protein

Table 5. Experiments with Wine Protein Fractions A (Thaumatin-like) and I (Chitinase), Anion Additions to Model Wine Samples, and Anion Concentrations Reported in Grape Juice and White Wine

	amount added (mg/L)		amount reported (mg/L)		
anion	separate addition	mixture	range	mean	ref
acetate	1000	500	300–1000	–	*44*
chloride	200	200	up to 1148[a]	141[a]	*31*
			up to 400	–	*44*
citrate	1000	200	100 – 300	–	*44*
			up to 700	–	*45*
malate	–[b]	3000	>5000	–	*45*
phosphate	1000	800	50–900	–	*44*
			50–1000	–	*46*
sulfate	100, 200, 300, 400, 600,[c] 800,[c] 1000,[c] 100,[d] 400[d]	400	up to 850[a]	185[a]	*31*

[a] For juices. [b] Not used. [c] For wine thaumatin-like protein only. [d] For mixture of wine thaumatin-like protein and chitinase (each 75 mg/L).

purification schemes, although the levels employed are severalfold higher than those in wine. In the particular case of white wine, this loss of water of solvation, even by a relatively low amount of sulfate anion, by a protein in a solution containing a variety of cations and other anions and between 9 and 13% (v/v) ethanol may be enough to affect the tertiary structure and/or aggregation of proteins. Sulfate is regarded as a stabilizing precipitant (*33*), because tertiary bonds seldom are negatively affected during protein purification, at least at cold temperatures. However, it is possible that the combined effect of pH, relative to the p*I* of the protein in question, temperature, alcohol, and sulfate may yield an entirely different picture. We favor a role in denaturation as opposed to aggregation given that salts in general, including sulfate salts, tend to limit rather than promote aggregation (*34*). Nevertheless, the available data suggest that sulfate is both essential to and capable of modulating the intensity of haze of model wine solution. This notion now needs to be tested further to ascertain more clearly the relative importance of sulfate compared to other wine components.

A range of other variables (pH and ethanol in particular) are known to affect protein haze formation and may influence the effect of essential factors, but were not included in this study and require further consideration in this context. Siebert and colleagues (*35*, *36*) concluded that maximum haze in a model system that mimicked beer occurred at pH 4−4.5 when ethanol was 12%, with less haze at lower and higher pH values. Mesquita et al. (*11*) added extra alcohol to white wine (0.5, 1, and 2% v/v) and found alcohol had no influence. Another study using a model wine (*13*) came to similar conclusions regarding pH. However, Mesquita and colleagues (*11*) employed actual wine samples rather than a model wine to obtain results different from those above. The white wine they used became increasingly heat stable as the pH rose from 2.5 to 7.5, suggesting that pH does play an important role in protein haze formation and that a high pH reduces the potential to form protein haze in response to heat. However, it is not clear what the magnitude of importance of pH variation on haze level is within the range encountered in commercial white wine (2.9−3.6). Given that the pH range in commercial white wines is still at least 1 pH unit below the isoelectric points of the wine proteins, and the lack of evidence in the literature that pH in this range has a significant role in haze formation, all experimentation in this study was undertaken at a fixed pH of 3.0.

Although sulfate appears to be fundamental to haze formation, other wine components such as phenolic compounds remain as candidate haze modulators. One possibility is that white wine phenolic compounds affect the particle size of denatured aggregated proteins, possibly through cross-linking. Several researchers (*37*, *38*) have suggested a hydrophobic mechanism for the interaction between phenolic compounds and proteins, in which the protein has a fixed number of phenolic binding sites. More of these sites are exposed when the protein is denatured. PVPP fining, which results in the removal of phenolic compounds from wine, reduced protein haze levels (**Table 4**).

Protein haze in white wine thus differs in several aspects from protein haze in beer. It is well-established that beer protein haze is due to interactions between proteins, derived from the barley storage protein hordein, and rich in proline, and hop polyphenolic compounds (*36*, *39*−*41*). White wine proteins are not derived from storage proteins of grape seed, nor are they as rich in proline as hordein. In addition, wine protein haze formation cannot be eliminated by removing polyphenolic compounds by PVPP (**Table 4**), whereas in beer this has been applied as a commercial strategy (*42*, *43*).

It is clear that protein haze formation in white wine is a multifactorial process. This study elucidates one new factor that is involved, in addition to protein itself, and it is possible that other as yet unidentified wine components also participate in protein haze formation. Further searches for additional "unknown" components are needed as well as a multifactorial study toward investigating the interactions involved, even with components shown to be nonessential with the criteria applied in the present study. Identification of essential factors may ultimately yield novel methods to tackle protein haze formation that either reduce or abolish the need for bentonite. However, even if essential components are identified, their selective removal, as with wine proteins themselves, may not be achievable without unwanted side effects.

Nevertheless, we envisage that a more thorough understanding of the mechanisms and participants in protein haze formation may lead to new technologies to control this potential quality defect in wine and may lead to more efficient predictive tools based on rapid compositional analysis.

ACKNOWLEDGMENT

We thank Steven Van Sluyter of the Australian Wine Research Institute for fruitful discussions regarding this work and Millipore Waters (Australia) for the gift of the mixed-mode cartridges.

LITERATURE CITED

(1) Waters, E. J.; Alexander, G.; Muhlack, R.; Pocock, K. F.; Colby, C.; O'Neill, B. N.; Høj, P. B.; Jones, P. R. Preventing protein haze in bottled wine. *Aust. J. Grape Wine Res.* **2005**, *11*, 215−225.

(2) Waters, E. J.; Shirley, N. J.; Williams, P. J. Nuisance proteins of wine are grape pathogenesis-related proteins. *J. Agric. Food Chem.* **1996**, *44*, 3−5.

(3) Siebert, K. J. Effects of protein−polyphenol interactions on beverage haze, stabilization, and analysis. *J. Agric. Food Chem.* **1999**, *47*, 353−362.

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

(5) Somers, T. C.; Ziemelis, G. Direct determination of wine proteins. *Am. J. Enol. Vitic.* **1973**, *24*, 47−50.

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

(7) Yokotsuka, K.; Nozaki, K.; Kushida, T. Turbidity formation caused by interaction of must proteins with wine tannins. *J. Ferment. Technol.* **1983**, *61*, 413−416.

(8) Waters, E. J.; Peng, Z.; Pocock, K. F.; Williams, P. J. Proteins in white wine, I: Procyanidin occurrence in soluble proteins and insoluble protein hazes and its relationship to protein instability. *Aust. J. Grape Wine Res.* **1995**, *1*, 86−93.

(9) Besse, C.; Clark, A.; Scollary, G. Investigation of the role of total and free copper in protein haze formation. *Aust. Grapegrower Winemaker* **2000**, *437*, 19−20.

(10) Pellerin, P.; Waters, E.; Brillouet, J.-M.; Moutounet, M. Effet de polysaccharides sur la formation de trouble proteique dans un vin blanc (Effect of polysaccharides on protein haze formation in white wine). *J. Int. Sci. Vigne Vin* **1994**, *28*, 213−225.

(11) Mesquita, P. R.; Piçarra-Pereira, M. A.; Monteiro, S.; Loureiro, V. B.; Teixeira, A. R.; Ferreira, R. B. Effect of wine composition on protein stability. *Am. J. Enol. Vitic.* **2001**, *52*, 324−330.

(12) Doco, T.; Vuchot, P.; Cheynier, V.; Moutounet, M. Structural modification of wine arabinogalactans during aging on lees. *Am. J. Enol. Vitic.* **2003**, *54*, 150−157.

(13) Fenchak, S. F.; Kerr, W. L.; Corredig, M. Multifactorial study of haze formation in model wine systems. *J. Food Qual.* **2002**, *25*, 91−10.

(14) Waters, E. J.; Pellerin, P.; Brillouet, J.-M. A *Saccharomyces* mannoprotein that protects wine from protein haze. *Carbohydr. Polym.* **1994**, *23*, 185−191.

(15) Waters, E. J.; Pellerin, P.; Brillouet, J.-M. A wine arabinogalactan-protein that reduces heat-induced wine protein haze. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 43−48.

(16) Waters, E. J.; Wallace, W.; Tate, M. E.; Williams, P. J. Isolation and partial characterization of a natural haze protective factor from wine. *J. Agric. Food Chem.* **1993**, *41*, 724−730.

(17) Vidal, S.; Francis, L.; Guyot, S.; Marnet, N.; Kwiatkowski, M.; Gawel, R.; Cheynier, V.; Waters, E. J. The mouth feel properties of grape and apple proanthocyanidins in a wine like medium. *J. Sci. Food Agric.* **2003**, *83*, 564−573.

(18) Waters, E. J.; Peng, Z.; Pocock, K. F.; Williams, P. J. Proteins in white wine, II: Their resistance to proteolysis is not due to either phenolic association or glycosylation. *Aust. J. Grape Wine Res.* **1995**, *1*, 94−99.

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

(20) Hayasaka, Y.; Adams, K. S.; Pocock, K. F.; Baldock, G. A.; Waters, E. J.; Hoj, P. B. Use of electrospray mass spectrometry for mass determination of grape (*Vitis vinifera*) juice pathogenesis-related proteins: a potential tool for varietal differentiation. *J. Agric. Food Chem.* **2001**, *49*, 1830−1839.

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

(22) Iland, P. G.; Ewart, A. J. W.; Sitters, J. H.; Markides, A. J.; Bruer, N. G. C. *Techniques for Chemical Analysis and Quality Monitoring during Winemaking*; Patrick Iland Wine Promotions: Campbelltown, South Australia, 2000; p 43.

(23) Cozzolino, D.; Kwiatkowski, M. J.; Parker, M.; Cynkar, W. U.; Dambergs, R. G.; Gishen, M.; Herderich, M. J. Prediction of phenolic compounds in red wine fermentations by visible and near infrared spectroscopy. *Anal. Chim. Acta* **2004**, *513*, 73−80.

(24) Somers, T. C.; Ziemelis, G. Spectral evaluation of total phenolic components in *Vitis vinifera*: grapes and wines. *J. Sci. Food Agric.* **1985**, *36*, 1275−1284.

(25) Pocock, K. F.; Rankine, B. C. Heat test for detecting protein instability in wine. *Aust. Wine, Brew. Spirit Rev.* **1973**, *91 (5)*, 42−43.

(26) Henschke, P.; Jiranek, V. Yeasts. Metabolism of nitrogen compounds. In *Wine Microbiology and Biotechnology*; G. H. Fleet, Ed.; Harwood Academic Publishers: Chur, Switzerland, 1993.

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

(28) Fuleki, T.; Ricardo-Da-Silva, J. M. Effects of cultivar and processing method on the contents of catechins and procyanidins in grape juice. *J. Agric. Food Chem.* **2003**, *51*, 640−646.

(29) Gorinstein, S.; Caspi, A.; Zemser, M.; Trakhtenberg, S. Comparative contents of some phenolics in beer, red and white wines. *Nutr. Res.* **2000**, *20*, 131−139.

(30) Pyrzynska, K. Analytical methods for the determination of trace metals in wine. *Crit. Rev. Anal. Chem.* **2004**, *34*, 69−83.

(31) Leske, P. A.; Sas, A. N.; Coulter, A. D.; Stockley, C. S.; Lee, T. H. The composition of Australian grape juice: chloride, sodium and sulfate ions. *Aust. J. Grape Wine Res.* **1997**, *3*, 26−30.

(32) Kunz, W.; Henle, J.; Ninham, B. W. Zur Lehre von der Wirkung der Salze (about the science of the effect of salts): Franz Hofmeister's historical papers. *Curr. Opin. Colloid Interface Sci.* **2004**, *9*, 19−37.

(33) Creighton, T. E. *Proteins: Structures and Molecular Properties*; W. H. Freeman: New York, 1993; p 507.

(34) Kita, Y.; Arakawa, T. Salts and glycine increase reversibility and decrease aggregation during thermal unfolding of ribonuclease-A. *Biosci., Biotechnol., Biochemistry* **2002**, *66*, 880−882.

(35) Siebert, K. J.; Carrasco, A.; Lynn, P. Y. Formation of protein−polyphenol haze in beverages. *J. Agric. Food Chem.* **1996**, *44*, 1997−2005.

(36) Siebert, K. J.; Lynn, P. Y. Effects of alcohol and pH on protein−polyphenol haze intensity and particle size. *J. Am. Soc. Brew. Chem.* **2003**, *61*, 88−98.

(37) Oh, H. I.; Hoff, J. E.; Armstrong, G. S.; Haff, L. A. Hydrophobic interaction in tannin−protein complexes. *J. Agric. Food Chem.* **1980**, *28*, 394−398.

(38) Siebert, K. J.; Troukhanova, N. V.; Lynn, P. Y. Nature of polyphenol−protein interactions. *J. Agric. Food Chem.* **1996**, *44*, 80−85.

(39) Miedl, M.; Garcia, M. A.; Bamforth, C. W. Haze formation in model beer systems. *J. Agric. Food Chem.* **2005**, *53*, 10161−10165.

(40) Siebert, K. Protein−polyphenol haze in beverages. *Food Technol.* **1999**, *53*, 54−57.

(41) Bamforth, C. W. Beer haze. *J. Am. Soc. Brew. Chem.* **1999**, *57*, 81−90.

(42) Leiper, K. A.; Stewart, G. G.; McKeown, I. P.; Nock, T.; Thompson, M. J. Optimising beer stabilisation by the selective removal of tannoids and sensitive proteins. *J. Inst. Brew.* **2005**, *111*, 118−127.

(43) Madigan, D.; Byrne, H.; Matthews, S.; Kelly, R.; McEnroe, C.; Harmey, D. Studies on the effects of common process variables on the colloidal stability of beer. *J. Am. Soc. Brew. Chem.* **2000**, *58*, 160−164.

(44) Amerine, M. A.; Berg, H. W.; Kunkee, R. E.; Ough, C. S.; Singleton, V. L.; Webb, A. D. *The Technology of Wine Making*, 4th ed.; Avi Publishing: Westport, CT, 1980.

(45) Amerine, M. A.; Ough, C. S. *Methods for Analysis of Musts and Wines*; Wiley: New York, 1980.

(46) Rankine, B. C. *Making Good Wine*; Pan Macmillan: Sydney, Australia, 1989.

Received for review September 18, 2006. Revised manuscript received December 7, 2006. Accepted January 4, 2007. This work was supported by Australia's grape growers and winemakers through their investment body, the Grape and Wine Research and Development Corporation, with matching funds from the Australian government, and by the Commonwealth Cooperative Research Centres Program.

JF062658N