Protein evolution during the early stages of white winemaking and its relations with wine stability

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Abstract

Background and Aims: Grape proteins are responsible for the appearance of haziness in white wines during storage after bottling. However, only a few studies have approached the analysis of the fate of must proteins throughout the alcoholic fermentation. This study aimed to systematically investigate the daily variations in protein type and content during the fermentation in order to understand its influence on hazing potential and to attain some basic information to improve the practical management of grape proteins involved in the hazing of white wines.

Methods and Results: The evolution of total soluble protein and individual protein fractions was studied in samples taken before, during and after alcoholic fermentation of a white grape must. The results were then related to variations in protein instability as measured by the heat test. Both the quantity of soluble protein and the protein instability increased during fermentation and then decreased after 1-month storage of the wine. Protein composition did not vary during fermentation as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and anion exchange chromatography (AEC). However, variations in the relative proportions of the six protein fractions obtainable by AEC were noted in the different samples. The contribution of each AEC protein fraction to wine instability was determined by considering both the intrinsic instability and the relative quantity of each of the individual protein fractions in the wine. It was demonstrated that the grape thaumatin-like protein VVTL1, as identified by mass spectrometry, showed the largest increase during fermentation and accounted for almost 40% of the heat-induced haze of the final wine. Moreover, the decreased protein instability noted after one month storage of the wine could be attributed to the stabilizing effect of polysaccharides released by the yeast cells.

Conclusions: The quantity and relative proportion of soluble proteins vary during and after the alcoholic fermentation, as does their heat instability in wine. Grape VVTL1, constituting a large proportion of the total proteins in wine, seems to play a major role in protein haze formation. The release of yeast polysaccharides is related to an increased heat stability of total wine protein, despite the increase in the relative proportion of their most unstable component VVTL1. Therefore, the hazing potential of a white wine seems to be affected by variations in the relative proportions of its macromolecular components occurring in the early stages of winemaking.

Significance of the Study: This study addressed for the first time the issue of the protein changing during the fermentation of white wine. The results obtained here offer useful information to aid understanding of the contribution of individual proteins to white wine instability, which can be applied for the improvement of the winemaking process.

Abbreviations

AEC anion-exchange chromatography; **KDS** potassium dodecyl sulfate; **MS** mass spectrometry; **MW** molecular weight, **PAS** periodic acid-Schiff stain, **PR-proteins** pathogenesis-related proteins, **RT** retention time, **SDS-PAGE** sodium dodecyl sulfate-polyacrylamide gel electrophoresis, **TL** thaumatin-like, **VVTL1** *Vitis vinifera* thaumatin-like protein

Keywords: *chitinases, fermentation, haze, heat test, must, proteins, stability, thaumatin-like protein, white wine*

Introduction

The presence of haze in bottled white wines is a serious quality defect as turbidity makes a wine undesirable for consumers. Wine proteins, which have the tendency to become insoluble during wine storage, are the main cause of this defect (Bayly and Berg 1967, Hsu et al. 1987, Waters et al. 1992). However, the hazing potential of a wine does not seem to be correlated with its total protein concentration (Bayly and Berg 1967), suggesting a differential contribution of individual proteins to the phenomenon of haze formation (Hsu et al. 1987, Waters

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et al. 1992). Proteins present in wines are considered to be a mixture of molecules derived both from the grape berries and the yeast (Hsu et al. 1987). Fractions of berry-derived proteins remaining in wine belong to two main classes of pathogenesis-related (PR) proteins, namely chitinases (PR-3) and thaumatin-like (TL) proteins (PR-5), which are usually resilient to the winemaking process but are involved in hazing during wine storage (Waters et al. 1992, 1996, Tattersall et al. 1997). A recent study regarding the natural haze in a Sauvignon Blanc wine showed that, besides TL proteins and chitinases, other minor grape proteins like β-1,3-glucanase and ripening-related proteins can be involved in wine haze formation (Esteruelas et al. 2009). Yeast can also affect wine protein composition. They do so directly by releasing cellular components such as mannoproteins, which have a stabilizing effect on wine hazing (Waters et al. 1994, Moine-Ledoux and Dubourdieu 1999, Dupin et al. 2000) or indirectly by secreting extracellular proteases that may contribute to the hydrolysis of the berry proteins (Lagace and Bisson 1990, Dizy and Bisson 2000). However, it has been shown that berry-derived proteins in the wine have a high resistance to the action of the fermenting yeast (Waters et al. 1992, 1995).

Despite significant advances in wine protein research, very few studies have been devoted to the analysis of the fate of berry-derived proteins initially present in the juice as they evolve during the winemaking process. Of these studies, some found a decrease in total protein concentration (Hsu et al. 1987, Canals et al. 1998, Luguera et al. 1998, Dizy and Bisson 2000, Manteau et al. 2003), while others reported an increase in soluble protein content after alcoholic fermentation (Bayly and Berg 1967, Dizy and Polo 1996, Fukui and Yokotsuka 2003), which may be linked to proteins released from yeast cells. However, in most of these studies, variations were detected by comparing the protein contents before (in must) and after alcoholic fermentation (in wine), without reference to the evolution of protein quantity and type during that process.

A better knowledge of these aspects is needed in order to attain the basic information necessary to improve the practical management of grape proteins involved in hazing of white wines.

With the aim of assessing variations in protein quantity and composition during the early stages of winemaking, proteins were analysed using chromatographic, electrophoretic and mass spectrometry techniques. Moreover, these data were related to the thermal stability of the wine proteins by assessing the contribution of each individual protein fraction to total hazing potential of the wine.

Materials and methods

Wine preparation

Approximately 100 kg of grapes of *Vitis vinifera* (white cv. Manzoni Bianco) were pressed with a pneumatic press (Provintec s.n.c. Kellertartikel, Bolzano, Italy) at ≤2 atm. Before fermentation the must was treated with SO_2 (50 mg/L) and settled by treatment with pectolytic enzymes (Everzym MPL, Ever, Pramaggiore, Italy) for 24 h at 4°C. After racking and addition of a selected *Saccharomyces cerevisiae* strain (Anchor VIN13, Ever, Pramaggiore, Italy), fermentation took place in stainless steel tanks (100 L) at 15–18°C. Fermentation was completed after 7 days with two rackings being carried out after 10 days (SO_2 was added to 25 mg/L of free SO_2) and 29 days after the end of fermentation, respectively. Samples were taken before fermentation (before and after enzymatic settling), during fermentation (every day) and after each of the two rackings.

Analytical methods

Titratable and volatile acidity, pH, reducing sugar and alcohol content and pH, were determined following the official methods of analysis proposed by the Office International de la Vigne et du Vin (O.I.V).

Polyphenol and polysaccharide content determination

Total polyphenol content was measured by using the Folin–Ciocalteu colorimetric method (Singleton and Rossi 1965). The results were expressed in mg/L of gallic acid. Total polysaccharide content was determined following the procedure described by Segarra et al. (1995) and results were expressed in mg/L of glucose.

Protein content determination

Protein content was determined using the Potassium dodecyl sulphate (KDS) method (Vincenzi et al. 2005). First, proteins were precipitated from 1 mL of wine by adding 10 µL of 10% (w/v) Sodium dodecyl-sulphate (SDS) and 250 µL of 1 M KCl. The pellets obtained after centrifugation were then dissolved in 1 mL of distilled water and protein was quantified using a bicinconinic acid (BCA) 200 protein assay kit (Pierce, Rockford, Illinois, USA) according to the manufacturer's instructions. The calibration curve was prepared by using a serial dilution in water of bovine serum albumin (Sigma, Milan, Italy) ranging from 62.5 µg/mL to 1000 µg/mL.

Total protein preparation

Musts and wines were dialysed against distilled water in 3.5 kDa cut off dialysis bags (Spectra/Por3, Spectrum, Los Angeles, California, USA) and passed onto a Solid Phase Extraction C-18 cartridge (1 mL resin, Supelco, Inc, Bellefonte, Pennsylvania, USA) to separate the protein extract from residual polyphenols (Waters et al. 1992). Afterwards, the obtained preparations were freeze-dried and dissolved in a small volume of water for long-term storage at −20°C.

Anion Exchange Chromatography (AEC)

The chromatographic separations were performed on a high-performance liquid chromatography (HPLC) apparatus (Waters 1525) equipped with a Dual λ Absorbance Detector (Waters 2487). The collected data were processed using Waters Breeze™ Chromatography Software (Version 3.30, Waters, Milford, Massachusetts, USA). Total protein preparations from must and wine samples were solubilized in 20 mM Tris-HCl pH 8.5 (eluant A) and loaded onto a Resource™ Q column (Amersham Biosciences, Uppsala, Sweden) equilibrated with the same buffer at a flow rate of 1 mL/min. Bound proteins were eluted with a gradient of eluant B (eluant A containing 1 M NaCl) as follows: 0% to 14% of eluant B in 70 min, to 50% eluant B in 20 min and finally to 100% eluant B in 5 min and held for 15 min. Protein peaks were collected, dialysed and freeze-dried.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic analyses were performed according to Laemmli (1970). For SDS-PAGE in reducing conditions, 3% (v/v) of 2-mercaptoethanol was added to the loading buffer. The molecular weight standard proteins were Broad Range Molecular Weight Markers (Bio-Rad Laboratories, Richmond, Califor-

nia, USA). Gels were stained with Coomassie Brilliant Blue R-250 and destained with 7% acetic acid. Glycoproteins were stained with the periodic acid-Schiff (PAS) reagent (Sigma, Milan, Italy) according to the manufacturer's instructions.

Reverse Phase (RP) – HPLC

The protein composition of AEC-HPLC wine protein fractions was determined by RP-HPLC using a C-18 column (Vydac 218 MS 54, Hesperia, California, USA) according to the method of Peng et al. (1997). Proteins were tentatively identified by comparison of their retention times to those of purified grape PR proteins (Marangon et al. 2009).

Heat test

Solutions of both the total protein (300 mg/L) and individual AEC-HPLC fractions (150 mg/L) in ultrafiltered (3.5 kDa cut off) wine were heated at 80°C for 6 h and then cooled to 4°C for 16 h. Hazing was then measured by calculating the difference in absorbance at 540 nm before and after heating of the sample (Waters et al. 1992).

Protein identification

After protein quantification, the sample was diluted to a concentration of 1 mg/mL in 50 mM NH_4HCO_3, reduced with 10 mM Dithiothreitol (DTT) (1 h, 37°C, dark) and alkylated with 30 mM iodoacetamide (30 min, room temperature, dark). Sequencing grade modified trypsin (Promega, Madison, Wisconsin, USA) was added at an enzyme:protein ratio of 1:50 (w/w) for digestion overnight at 37°C. The digested protein was mixed with an equal volume of matrix solution (α-cyano-4-hydroxycinnamic acid, 5 mg/mL in 70% acetonitrile, 0.1% TFA) and 1 μL was spotted on a 384-well AB OptiTOF MALDI stainless steel target plate. Sample was analysed using a MALDI-TOF/TOF 4800 Analyzer (Applied Biosystems, Toronto, Canada) with 4000 Series Explorer v3.5.3 software. Mass spectrometry (MS) data was acquired automatically over a mass range of 900–3500 Da in the positive-ion reflector mode. In the MS spectrum, the 10 most abundant MS peaks were selected for MS/MS. MS/MS data were searched using the Mascot search engine (Matrix Science, London, UK) against the MSDB database (3239079 sequences; 1079594700 residues; Taxonomy: Viridiplantae (247880 sequences)). Enzyme specificity was set to trypsin with one missed cleavage using a mass tolerance window of 50 ppm for the precursor ion and 0.3 Da for the fragment ions and carbamidomethylcysteine as fixed modification.

Results and discussion

The must obtained from grapes of *Vitis vinifera* cv. Manzoni Bianco showed a turbidity of 181 NTU (nephelometric turbidity unit), which is considered to be in the range for optimal alcoholic fermentation (100–250 NTU, Ribéreau-Gayon et al. 2006). Must and wine samples were taken at different stages of the vinification. Musts were collected before (sample M) and after (sample M_P) overnight treatment with pectolytic enzymes. Wine samples were collected during (samples F1 to F6) and after fermentation (samples R1 and R2, the first and second rackings, respectively). Analytical data for these samples are reported in Table 1. At the time of the second racking, volatile acidity was 0.23 g/L, in agreement with usual values for a regular alcoholic fermentation (0.24–0.37 g/L, Ribéreau-Gayon et al. 2006). The low level of volatile acidity should indicate the absence of acetic bacteria activity. This finding is important because acetic bacteria possess the ability to produce extracellular proteases able to degrade wine proteins (Bossi et al. 2006).

Total polysaccharides showed a large decrease (46%) after must settling (Table 1), presumably because of polygalacturonase and pectinlyase activities (Lao et al. 1996). From that point on, the ethanol-insoluble polysaccharide content slowly increased with some fluctuation during the rest of the experiment, likely because of the complex phenomena of precipitation/solubilisation (Guadalupe and Ayestarán 2007). Release of yeast glucans and mannoproteins, that has been demonstrated to occur during the alcoholic fermentation (Gonçalves et al. 2002), could also contribute to total must/wine polysaccharides.

Because polyphenols, and in particular tannins, have the capacity to bind proteins and polysaccharides, leading to the formation of haze and sediments, the total polyphenol content was also measured. While pectolytic enzyme treatment caused only a low reduction in total polyphenols, a decrease of about 18% in the polyphenol content was noted after 1 day of fermentation (F1, Table 1), probably because of adsorption of these compounds to the cell wall of the fermenting yeast (Castino 1982). However, in the following days, the polyphenol content increased again to approximately 90% of the initial concentration in the must. This increase was presumably linked to desorption from the yeast cells under the rising ethanol concentration.

Table 1. Analytical data for the samples collected before (M, must, M_p, must after treatment with pectolytic enzymes) during (F1 to F6) and after (R1 and R2) the alcoholic fermentation.

Sampling	pH	Titratable acidity (g/L tartaric acid)	Reducing sugars (% w/v)	Ethanol (% v/v)	Polysaccharides (mg/L)	Polyphenols (mg/L)
M (day 0)	3.32	7.0	22.9	n.d.	1061 ± 15	202.9 ± 6.7
M_P (day 1)	3.26	7.0	24.1	n.d.	584 ± 9	191.8 ± 5.6
F1 (day 2)	3.22	7.3	18.2	n.d.	713 ± 13	156.9 ± 0.6
F2 (day 3)	3.21	7.6	14.7	n.d.	748 ± 18	171.1 ± 2.6
F3 (day 4)	3.20	7.8	9.6	n.d.	931 ± 10	182.8 ± 1.2
F4 (day 5)	3.23	7.5	3.2	n.d.	697 ± 7	180.1 ± 1.5
F6 (day 7)	3.25	7.2	<1	13	600 ± 16	185.6 ± 4.9
R1 (day 17)	3.21	7.1	n.d.	13.4	805 ± 14	186.5 ± 2.9
R2 (day 36)	3.22	7.2	n.d.	13.3	720 ± 11	174.5 ± 1.6

Days after harvest are indicated in brackets. All values are a mean of three measurements. n.d., not determined.

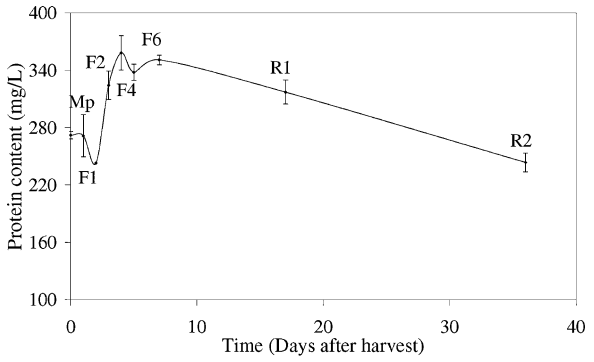

Figure 1. Variation in the total protein content (as mg/L of bovine serum albumin) in the samples collected at the different times of the experiment.

In addition to slight variations in the polysaccharide and phenolic contents, changes in the protein concentration of the samples during the experiment were also noted. The quantification assay applied in this case was the KDS method, that was shown to be reliable for measurement of protein content in the presence of interfering substances such as polyphenols and sugars (Vincenzi et al. 2005). The protein content measured in our case was high if compared with concentrations found in other wine samples (Dizy and Polo 1996, Luguera et al. 1998, Dizy and Bisson 2000, Ferreira et al. 2000, Fukui and Yokotsuka 2003). This is partially attributable to the naturally high protein content of Manzoni Bianco wine (Vincenzi and Curioni 2005, Vincenzi et al. 2005), and in part to the underestimation of the true value of protein concentration with the Bradford assay (Waters et al. 1991) which is widely used for wine protein quantification. After an initial drop, protein content increased by almost 30% in samples collected daily during the alcoholic fermentation, varying from 270 mg/L in the must to 350 mg/L in the wine at the end of the fermentation process (Figure 1). This protein increase may be explained by both yeast protein release and further protein solubilisation from suspended grape particles. As previously mentioned, studies on the changes in grape-derived proteins in the early stages of winemaking showed that quantitative changes occur during alcoholic fermentation, resulting either in a decreased (Hsu et al. 1987, Canals et al. 1998, Luguera et al. 1998, Dizy and Bisson 2000, Manteau et al. 2003) or increased protein content (Bayly and Berg 1967, Dizy and Polo 1996, Fukui and Yokotsuka 2003) in the fermented wine. Some authors also found qualitative protein changes during fermentation, with the appearance/disappearance of some protein components (Bayly and Berg 1967, Hsu et al. 1987, Canals et al. 1998, Ferreira et al. 2000). Conversely, in our case the electrophoretic analysis of samples taken before, during and after fermentation (Figure 2) did not show the appearance of new protein bands as reported previously (Luguera et al. 1998, Ferreira et al. 2000), indicating little or no contribution of yeast proteins.

During the post-fermentation period, the protein content slowly decreased to a point that at the second racking reflected that measured in the must (Figure 1). This may be partially explained by protein insolubilisation and precipitation, although the activity of proteolytic enzymes released from yeast after the end of the fermentation cannot be excluded (Lagace and Bisson 1990, Dizy and Bisson 2000).

The SDS-PAGE analysis under reducing conditions of the soluble proteins contained in must and wine samples revealed only four bands with apparent MWs of ≈ 60, 32, 24 and 14 kDa

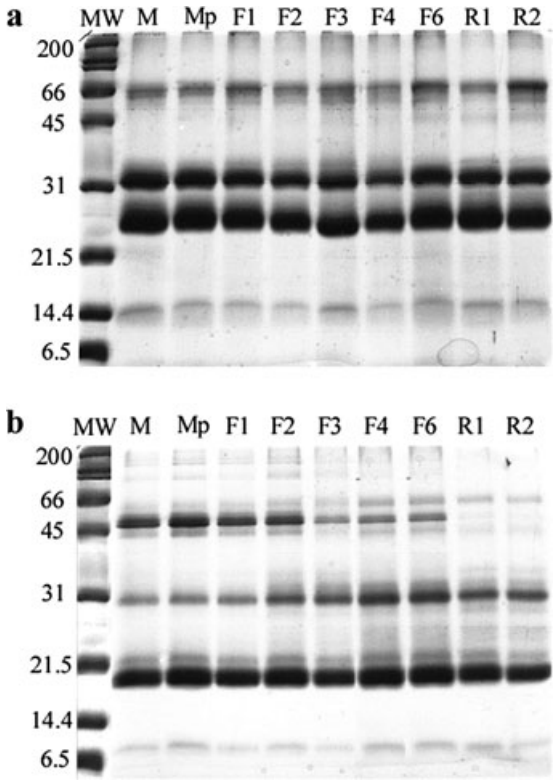

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis in reducing (a) and non-reducing (b) conditions of the soluble proteins of the samples collected at the different times of the experiment. Each lane contains the protein quantity present in 100 μL of sample. (MW, molecular weight standard proteins.)

(Figure 2a). Based on their SDS-PAGE mobility and relative abundance, the two major bands of 32 and 24 kDa have been considered to correspond to chitinases (Derckel et al. 1996, Vincenzi and Curioni 2005) and TL proteins (Tattersall et al. 1997) of the grape berry, respectively, which are well known to be involved in wine protein instability (Waters et al. 1996). A different SDS-PAGE pattern was obtained under non-reducing conditions (Figure 2b). This effect probably is because of the lower hydrodynamic volume of the proteins when stabilised by intra-molecular disulphide bonds (Vincenzi and Curioni 2005). While all samples under non-reducing conditions contained the same protein bands, there were variations in the relative intensity of some of them (Figure 2b). In particular, the staining intensity of the band at ≈ 30 kDa in the unreduced samples showed an increase during fermentation, whereas the band at ≈ 50 kDa decreased in the same samples, becoming only barely detectable after 10 days of wine storage (Figure 2b). These variations were not noticeable when the proteins were analysed under reducing conditions (Figure 2a).

By comparison with the results previously obtained by staining the SDS-PAGE gels for chitinolytic activity detection in grape and wine, the unreduced ≈ 30 kDa band can be tentatively identified as the main grape chitinase isoform (a class IV chitinase), which migrates to an apparent MW of ≈32 kDa when reduced (Waters et al. 1992, Vincenzi and Curioni 2005). Proteins with MW of ≈50 kDa in non-reducing SDS-PAGE were recently attributed to chitinases (Marangon et al. 2009). The presence of the ≈50 kDa band only in the unreduced samples (compare Figure 2a and 2b), and its decrease with the time course experiment in correspondence

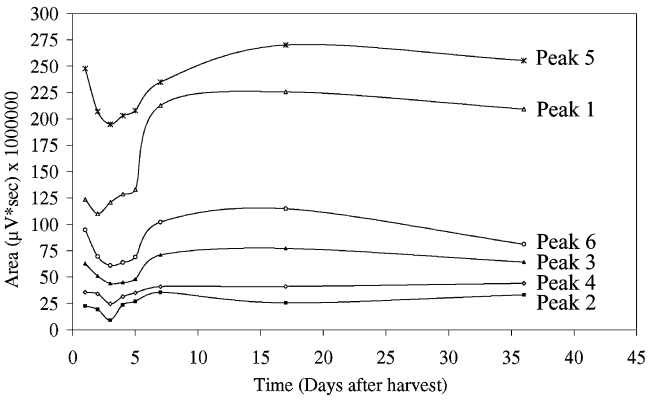

Figure 3. Variation of the anion exchange chromatography peak areas for the samples collected at the different times of the experiment.

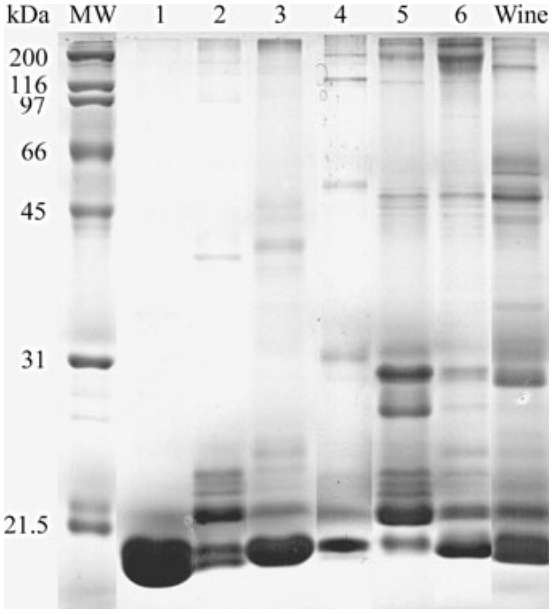

Figure 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis in non-reducing conditions of the peaks collected from anion exchange chromatography (AEC) of the proteins of the sample taken at the end of fermentation. Fifteen μg of protein were loaded in each lane. (Wine, total proteins of the wine before AEC fractionation; MW, molecular weight standard proteins.)

with the increase of the ≈ 30 kDa chitinase band, suggests a relation between the two bands which is affected by the reducing conditions. To determine whether the ≈ 30 kDa band was derived from the reduction of the ≈ 50 kDa band, the latter (from a must sample) was excised from a non-reduced SDS-PAGE gel and loaded on a new gel after being reduced. This second gel showed only a band at ≈ 30 kDa (data not shown), indicating that the ≈50 kDa band was a disulphide-linked aggregate containing the ≈30 kDa band. Therefore the only qualitative change in protein content detected during the experiment (namely the disappearance of the ≈50 kDa band, Figure 2b) was likely related to a decrease in the red-ox potential of the wine, which may have been caused by the activity of the yeast (Kukec et al. 2002).

A good separation of grape and wine proteins was also achieved by anion exchange chromatography (AEC), as reported by other authors (Waters et al. 1992, Dorrestein et al. 1995, Monteiro et al. 2001). In order to remove polyphenols, samples were passed through a C-18 cartridge. This operation may result in a certain quantitative protein loss, but it does not affect the protein composition of the sample (Waters et al. 1992). Six peaks were detected by AEC in the must. The same peaks were detected in all the must/wine samples collected during fermentation and storage but their relative proportions changed, as revealed by the quantification of the chromatographic areas (Figure 3). Throughout the experiment, trends of the peak areas were generally in congruence with that of total protein content (compare Figures 3 and 1). However, even though total protein content started to increase after the first day of fermentation (sample F1), the increase in the area of almost all the AEC peaks started after the second day of fermentation (sample F2). The behaviour of total protein content can be probably explained by that of Peak 1, whose area in fact started to increase from the first day of fermentation (Figure 3).

If the variations in the peak areas were because of the adsorption/desorption to/from suspended particles including yeast cells, the protein of Peak 1 should be the most highly involved in these processes, its behaviour likely being affected by variations in the characteristics of the solvent and, in particular, to variations in ethanol concentration. Therefore, the 30% increase in total protein content over the length of the experiment (Figure 1) should be because of the increase (+72%) of AEC Peak 1 (Figure 3). This peak was collected and analysed by Reverse-Phase RP-HPLC (data not shown), showing to contain 98% TL proteins (RT 8.95 min) and a trace amount of chitinases (RT 19.90 min), as assessed on the basis of retention time (Peng et al. 1997). To confirm the identity of the main Peak 1 protein, the latter was analysed by MS. The results indicated that this protein corresponded to VVTL1 (accession number gi|2213852|, peptides matched 10, sequence coverage 54%) the main *Vitis Vinifera* thaumatin-like protein (Tattersall et al. 1997).

The largest AEC peak (Peak 5) also increased from 35% to 42% during fermentation. RP-HPLC analysis revealed that this peak contained trace amounts of TL proteins (RT 8.95 min) and a large quantity of chitinases (RT 19.90 min) (Peng et al. 1997), which is in agreement with previous findings obtained by chitinase activity detection on SDS-PAGE gels (Vincenzi et al. 2007). These results confirm that TL proteins and chitinases are the main protein components in must and wine (Waters et al. 1992).

The six peaks obtained by AEC of the wine proteins were analysed at the same protein concentration by SDS-PAGE (Figure 4). Most of the bands of the different AEC fractions were not present or barely detectable on the SDS-PAGE profile of total wine proteins, because of their low relative quantity in the total protein mixture.

AEC Peak 1 showed only one band at ≈ 20 kDa, corresponding to the expected MW for the VVTL1 identified by MS in this fraction. In contrast, all the other AEC fractions contained two or more bands, including AEC fraction 5, which displayed only one main peak by RP-HPLC at a retention time corresponding to that of chitinases (Peng et al. 1997) (data not shown). The reason for the differences in the relative quantities of the different protein components of fraction 5 appearing in SDS-PAGE versus RP-HPLC analyses may be explained by the very low binding of the Coomassie dye to chitinase bands when SDS-PAGE is performed in non-reducing conditions, as noted previously (Vincenzi et al. unpublished data).

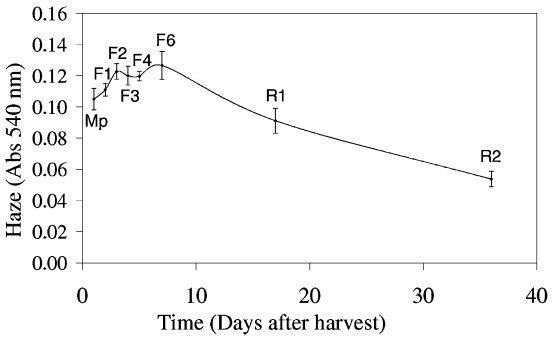

Figure 5. Haze formed after the heat-test by the total proteins (300 mg/L in ultrafiltered wine) of the samples collected at the different times of the experiment.

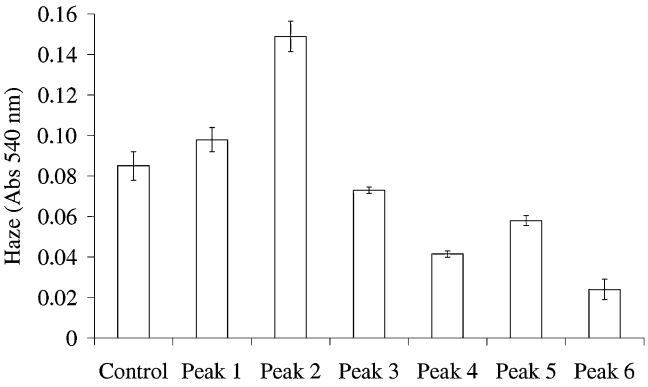

Figure 6. Haze formed after the heat-test by the proteins (150 mg/L in ultrafiltered wine) of the peaks collected from anion exchange chromatography of the sample taken at the end of fermentation. Control: total wine proteins (150 mg/L in ultrafiltered wine).

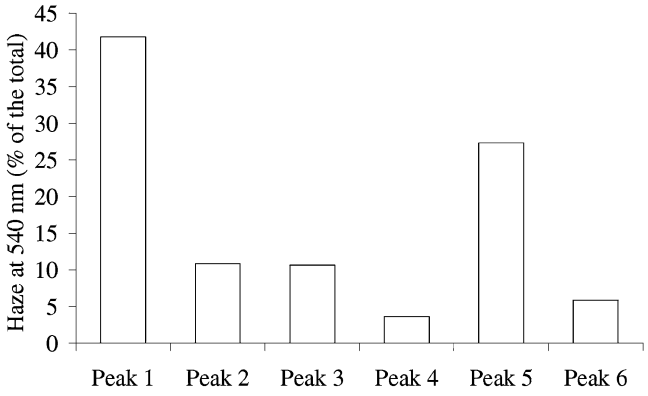

Figure 7. Percent contribution (calculated as intrinsic instability multiplied by relative concentration) of each anion exchange chromatography peak to the total turbidity developed by the heat-test in the sample taken at the end of fermentation.

Bands with similar apparent MWs at ≈ 20–22 kDa were present in almost every AEC peak (Figure 4), confirming the idea that wine contains a large number of polypeptides with different isoelectric points (affecting the elution time in AEC) but with similar apparent molecular masses in SDS-PAGE (Monteiro et al. 2001). These components derive directly from the berry, in which they are already present as a complex mixture of structurally similar polypeptides (Monteiro et al. 2007). This result agrees with the observed similarity of the AEC chromatograms obtained here for samples taken before and after fermentation (not shown).

Unlike the ≈20–22 kDa bands, the main chitinase isoform (≈ 30 kDa, see above) was present only in AEC fraction 5 (with a slight contamination of the fraction corresponding to peak 6) (Figure 4). Although several chitinase isoforms have been detected in grape (Derckel et al. 1996) and must (Waters et al. 1998), the results presented here suggest that the vinification process results in a simplification of the pattern of these enzymes. Indeed, only two chitinases have been detected in wine (Waters et al. 1996, Vincenzi and Curioni 2005), which in our case should correspond to the ≈ 30 and ≈ 28 kDa bands of AEC fraction 5 (Figure 4).

To evaluate variations in protein stability throughout the early stages of the vinification process, the total protein fractions of must and wine samples taken at the different times were heat-tested. It has been demonstrated that environmental conditions, and in particular those related to pH and ethanol concentration, affect protein stability in alcoholic beverages (Siebert 1999). Therefore, in order to avoid influences derived from the different compositions of the samples being compared (i.e. different concentrations of sugars and ethanol), the proteins obtained after dialysis and freeze-drying of each sample were dissolved in an ultrafiltered (3 kDa cut-off) wine at the same concentration (300 mg/L) before being heat-tested.

The heat-test results showed that total protein instability slowly increased through alcoholic fermentation, and progressively decreased afterward (Figure 5). The initial increase in protein instability may have been related to the quantitative variation of the different protein fractions, such as the increase in the TL protein of Peak 1, as observed by AEC (Figure 3). To test this hypothesis, the instability of the proteins of each individual AEC peak, all dissolved at 150 mg protein/L in ultrafiltered Manzoni Bianco wine, was determined by the heat-test. The total wine proteins (at the end of alcoholic fermentation) at the same concentration were used as the control. Only proteins contained in Peak 1 and 2 showed an intrinsic instability higher than that of a corresponding quantity of the total wine proteins (Figure 6). However, Peak 2 accounted only for a minimal part of the total wine proteins (5.1% of the total area of the chromatogram in the sample at the end of fermentation, Figure 3), thus contributing only slightly to the total instability of the wine. On the contrary, AEC Peak 1 accounted for more than 30% of the total wine proteins, being the second most abundant peak in the wine (Figure 3). Therefore, taking into account both the actual concentration in wine and the intrinsic protein instability of each peak, the protein of Peak 1 seems to be giving the greatest contribution (more than 40%) to total wine instability (Figure 7). Taking into account that this peak contained almost exclusively VVTL1 protein (Figure 4), the importance of this grape-derived component in wine hazing can be confirmed (Waters et al. 1992). Moreover, the fact that AEC Peak 1 showed the highest increase during fermentation (Figure 3) can explain, at least in part, the increased instability of total proteins passing from must to wine (Figure 5).

The proteins of Peak 5 also contributed to the total instability of the wine (Figure 7), this being more related to the large protein content of the peak (Figure 3) rather than to its intrinsic heat instability (Figure 6). This peak was found to contain several electrophoretic bands by SDS-PAGE analysis (Figure 4), of which one main component (≈ 30 kDa) was a chitinase. Moreover, also the band at ≈ 28 kDa (Figure 4) was likely to

correspond to a chitinase isoform as can be deduced from previous reports (Waters et al. 1996, 1998, Vincenzi and Curioni 2005, Marangon et al. 2009). Therefore, although the instability of Peak 5 can not be ascribed with certainty to a specific protein component, it is likely that the grape chitinases, which have been shown to contribute to wine protein hazing (Waters et al. 1996, 1998), are responsible for the turbidity caused by the proteins of that peak after the heat test.

After the end of fermentation, the instability of the total proteins tended to decrease and the heat-induced haze at the second racking was almost half of that of the starting must (Figure 5). This could not be ascribed to a modification in the relative concentration of the different proteins, because the ratios of the different protein peaks remained quite constant during the 1-month storage of the wine (Figure 3). Therefore, the increased protein stability may be attributed to a reduction in the total protein content of the wine, but also to the presence of stabilizing factors appearing over time. It is well accepted that yeast polysaccharides, specifically cell wall mannoproteins, have a protective effect against haze formation in white wines (Moine-Ledoux and Dubourdieu 1999, Dupin et al. 2000). As a matter of fact, PAS staining of SDS-PAGE gels of the different wine samples showed some increase in the quantity of glycocompounds in the area at the top of the gel (data not shown). This result is in agreement with observations that the polysaccharides released by yeast during fermentation are mainly mannoproteins with MWs between 100 kDa and 200 kDa (Llaubères et al. 1987). A band with an apparent MW of ≈ 45 kDa was also detectable by PAS staining, but only in the wine samples obtained at the first and, mainly, at second racking, this band being undetectable in the samples collected during the alcoholic fermentation (samples from F1 to F6). Since the yeast cells were not completely removed with the two rackings, the appearance of glycosylated compounds should indicate that some yeast autolysis started after 1 month of wine storage. As a matter of fact, the tendency to undergo autolysis has been shown to be dependent on the yeast strain and the storage conditions (Barcenilla et al. 2003) and the appearance of increasing quantities of mannoproteins at early stages of vinification has been reported recently (Guadalupe and Ayestarán 2007). Therefore, it is likely that the increased wine protein stability observed after the end of fermentation was related to the appearance of protective factors, possibly polysaccharides released by the yeast.

In conclusion, the results reported here indicate that the soluble proteins of the berry of a white grape variety vary during and after the alcoholic fermentation in both their quantity and relative proportion. The protein fraction of AEC Peak 1, constituted mainly of VVTL1, as demonstrated by MS identification, showed the lowest stability when individually heat-tested in ultrafiltered wine. This confirmed the major role in haze formation of TL proteins (Waters et al. 1996, 1998). The same protein fraction is that with the largest quantitative increase during fermentation, constituting a large proportion of the total proteins in wine. Taking into account that bands with similar SDS-PAGE mobility, corresponding to that of other members of the TL protein family, could also be detected in other AEC fractions, the peculiar characteristics of VVTL1 warrants further investigation. Moreover, the data reported here suggest that the release of polysaccharides or polysaccharide-containing constituents (probably mannoproteins) by the yeast already starts within the first month of vinification and corresponds to an improved heat-stability of total wine protein, despite the increase in the relative proportion of their most unstable component. Therefore, the hazing potential of a white wine seems to be affected by variations in the relative proportions of its macromolecular components occurring in the early stages of winemaking.

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