Identification of Heat-Unstable Wine Proteins and Their Resistance to Peptidases

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Two major wine proteins that contribute significantly to Muscat of Alexandria wine heat haze have been purified by anion-exchange chromatography. These proteins had M_rs of 32 000 and 24 000, and although the amino acid composition of each protein was distinct, both contained a high proportion of asparagine and/or aspartic acid, glycine, and either serine or threonine. The M_r 24 000 protein produced about 50% more haze than the M_r 32 000 protein per unit of weight. All of the wine proteins, and in particular the M_r 24 000 protein, were resistant to proteolysis at 15 °C. Such resistance was not due to the presence of inhibitors or wine polysaccharides acting as protective colloids, suggesting that wine proteins are naturally resistant to proteolytic degradation.

INTRODUCTION

There is a need to formulate alternative procedures to remove unstable haze-forming proteins from wines because the currently used technique of removal by adsorption onto bentonite is nonspecific and can impair the quality of wine. To find suitable alternative procedures, it is first necessary to determine the contribution that individual grape proteins make to heat-induced haze in order to target those proteins that are most susceptible to precipitation. It is thus appropriate to study the properties of these individual proteins so that more specific techniques for their removal can be developed. It is also appropriate that the source of these proteins be wines that are protein-rich and require treatment with high levels of bentonite for stabilization, such as those made from Muscat of Alexandria grapes.

One strategy to identify proteins that are involved in heat instability is to demonstrate haze prevention on removal of specific proteins and haze induction on back-addition of that material. The back-addition method was employed in a recent study in which crude protein fractions, dominated by either a M_r 32 000 or 24 000 protein, were found to be the most susceptible to heat-induced haze (Waters et al., 1991). These experiments also showed that the fraction containing the M_r 32 000 protein as a major component gave a heat-haze response approximately half that of fractions containing predominantly the M_r 24 000 protein. These data imply that the lower M_r proteins may be more important to wine haze. The first aim of the present study was the purification of these two proteins to homogeneity and confirmation of their different haze-forming potential.

The second part of this study concerns the application of peptidases as a technique to remove wine proteins through enzymatic degradation into small peptides and their component amino acids. This work on peptidases follows other studies in which juices and wines were treated with peptidase preparations at temperatures in excess of 30 °C, under which conditions a reduction in protein content was observed (Roklenko et al., 1980; Heatherbell et al., 1984; Lagace and Bisson, 1990). This elevated temperature requirement, however, precludes the use of such enzymes in premium winemaking in which juices and wines are generally held below 15 °C. In the work of Ngaba-Mbiakop (1981), where peptidases were used at 14 °C, the enzymes had little or no effect. This was not because the enzymes were inactive in grape juice and wine, since preliminary work in our laboratory demonstrated that five peptidase preparations were active in both juice and wine on model protein substrates such as casein and collagen at typical winemaking temperatures (Modra and Williams, 1988; Waters et al., 1990). However, even after prolonged incubation at 15 °C, treated wines did not show a significant decrease in protein concentration when compared to an untreated control wine (Waters et al., 1990), suggesting that grape and wine proteins were resistant to proteolytic attack.

The present research investigates the response to a commercial peptidase preparation of the most heat-unstable wine protein isolated in the first part of the study. The work confirms the proteolytic resistance of the wine protein and demonstrates that such resistance was not due to the presence of inhibitors or protective colloids.

MATERIALS AND METHODS

Materials. Muscat of Alexandria (synonym: Muscat Gordo Blanco) wines from the 1989–1991 vintages were used in this study. The wines, made from machine-harvested grapes grown in the Riverland, South Australia, were fermented to dryness and centrifuged prior to filtration through 30- and 20-S pads; no bentonite treatment was applied. The composition of the 1991 wine, which typified those used in the study, was as follows: protein concentration (by modified Bradford assay), 113 ppm of BSA equivalents; alcohol concentration, 12%; residual sugar, 2.2 g/L; free and total SO_2, 32 and 176 ppm; pH, 3.39; total titratable acidity, 7.7 g/L; volatile acidity, 0.37 g/L; copper, 0.2 ppm; iron, 0.3 ppm; calcium, 64 ppm; potassium, 1340 ppm; sodium, 149 ppm. Vinozym P was donated by Novo Laboratories Pty. Ltd., NSW, Australia. Water was purified by a Milli-Q reagent water system (Millipore Pty. Ltd., NSW, Australia). BSA and Coomassie Brilliant Blue R-250 were obtained from the Sigma Chemical Co., St. Louis, MO. The ultrafiltration apparatus with a YM-10 membrane, nominal cutoff of M_r 10 000, was purchased from Amicon Corp.

Ultrafiltration. Ultrafiltration of wine was performed in a 50 mL capacity stirred cell at 5 °C under nitrogen pressure. The filtrate was collected under a nitrogen blanket to minimize oxidation.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). Discontinuous SDS–PAGE was performed according to the method of Laemmli (1970). Samples

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were diluted at least 5-fold in sample buffer, boiled for 4 min, and then loaded into the wells. The electrophoresis sample buffer was prepared by combining water (425 μL), 0.5 M Tris-HCl (pH 6.8) (125 μL), glycerol (100 μL), 10% (w/v) sodium dodecyl sulfate (200 μL), 2-mercaptoethanol (100 μL), and 0.05% (w/v) bromophenol blue (50 μL). A Bio-Rad Mini-Protean II unit (Bio-Rad Laboratories Pty. Ltd., Australia) was used to run the gels at a constant voltage setting of 200 V until the bromophenol tracker dye was 5 mm from the bottom of the gel (usually 40 min).

After electrophoresis, the separated proteins were fixed and stained by incubating the gel in fixative (methanol/acetic acid/water 4:1:5) containing Coomassie Brilliant Blue R-250 (0.1%) for 30 min and then destaining with fixative. Alternatively, gels were fixed after electrophoresis by standing in fixative for 30 min and then stained for protein with the Bio-Rad silver stain kit according to the procedure of Merril et al. (1981), following the instructions provided with the kit. Bio-Rad molecular weight standards were used as M_r markers. The M_r values of unknown samples were calculated from the linear regression equation of log M_r vs mobility.

Protein Purification. $(NH_4)_2SO_4$ (0.47 g/mL) was added to wine to give 70% saturation and the precipitate collected by centrifugation (19000*g*, 2 h), suspended in 0.1 M Tris-HCl (pH 6.8) at 0 °C, and then concentrated and desalted by ultrafiltration using a Centricon 10 microseparation device. The solution was filtered through a Sep-Pak C-18 cartridge (Waters Associates, Milford, MA) to remove phenolic contaminants. The Sep-Pak had previously been activated with methanol (2 mL) and washed with water (5 mL). The final steps were undertaken on a Pharmacia FPLC system at room temperature. The sample was diluted 10-fold in 20 mM Tris-HCl (pH 8.0) and centrifuged (10000*g*, 5 min) before loading on a Mono-Q (HR 5/5) anion-exchange column which had been equilibrated with the above buffer. The NaCl concentration was increased from 0 to 500 mM over a period of 30 min. Peaks containing the M_r 24 000 and 32 000 proteins (as assessed by SDS–PAGE) were collected and rechromatographed as necessary to obtain both proteins in a purified state. Samples were concentrated and desalted using a Centricon 10 microseparation device. Purity was assessed by SDS–PAGE.

Determination of Haze Potential and Amino Acid Composition for Protein Quantification. Protein haze potential was determined by the procedures described in Waters et al. (1991). That paper also details the methods used for the acid hydrolysis of the protein samples and determination of the amino acids using reversed-phase HPLC with precolumn derivatization.

Peptidase Susceptibility. Vinozym P (5 g/L, 10 μL) was added to wine or ultrafiltered wine (1 mL) containing added protein (BSA, 500 μg; M_r 24 000 wine protein, 250 μg) and then incubated under nitrogen at 15 °C. The extent of hydrolysis occurring in these samples was assessed by discontinuous SDS–PAGE, as described above, with the following modifications to the sample preparation procedure to minimize artifacts. Samples (10 μL) were rapidly mixed with boiling electrophoresis sample buffer (40 μL), boiled for a further 4 min, and then placed in an ice bath for at least 5 min. No proteolytic action occurred during this procedure because even when BSA was preincubated in boiling electrophoresis sample buffer, to ensure the protein was denatured and thus potentially easily hydrolyzed by active peptidase, neither freshly prepared Vinozym P in electrophoresis sample buffer nor preboiled Vinozym P in electrophoresis sample buffer effected any proteolysis (data not shown). Samples that were not immediately analyzed were stored at −20 °C for a maximum of 2 weeks. After electrophoresis, the separated proteins were stained for protein with the Bio-Rad silver stain kit.

RESULTS AND DISCUSSION

Identification of Two Wine Proteins That Contribute to Wine Haze. (*a*) *Purification of Wine Proteins.* The separation of wine proteins on an FPLC Mono Q anion-exchange column at pH 8.0 is shown in Figure 1. A small amount of material was not bound to the exchange resin, but most of the material eluted as a group of partially resolved peaks. SDS–PAGE analysis (data not shown) of fractions collected throughout the chromatogram indicated that the first peak, eluting at 70 mM NaCl, contained a M_r 24 000 protein, the middle section, with at least three peaks eluting between 90 and 140 mM NaCl, contained proteins with M_rs 26 000 and 28 000, and then a M_r 32 000 protein eluted at 160 mM NaCl.

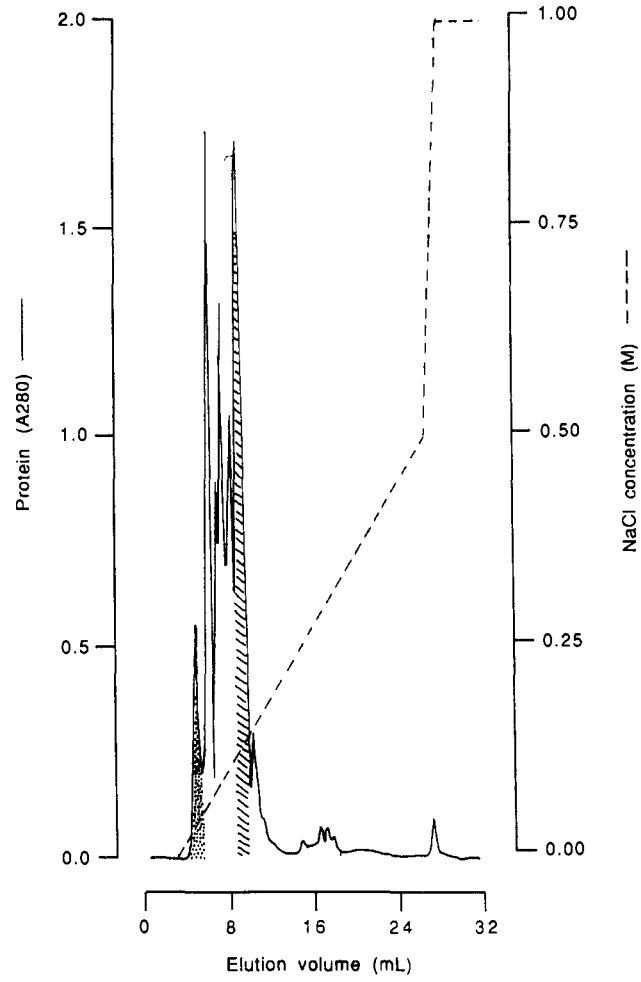

Figure 1. Separation of wine proteins by anion-exchange chromatography. The wine protein fraction precipitated by $(NH_4)_2SO_4$ (70% saturation) was fractionated on a FPLC-Mono Q column at pH 8.0 with a 0–0.5 M salt gradient (see Materials and Methods). The hatched regions were collected.

The 24 000 and 32 000 M_r proteins were of interest to this study because these were the major components in two earlier isolated crude fractions which contributed significantly to wine haze (Waters et al., 1991). Thus, a preparative chromatographic run was performed, and these proteins were collected. Their composition and purity were assessed by SDS–PAGE (Figure 2), which showed, by two staining procedures, that the samples selected for study were pure.

The yield of the purification and the protein concentrations of the M_r 24 000 and 32 000 proteins, as well as of the starting material (70% $(NH_4)_2SO_4$ ppt) before and after treatment to remove phenolic compounds by passage through a C-18 reversed-phase cartridge, are shown in Table I. This treatment, which removed material absorbing at 320 nm and diminished the 280-nm absorption, was necessary to preserve the life of the FPLC column, because phenolic compounds bind irreversibly to these high-resolution columns (Jervis and Pierpont, 1989). These results demonstrate that the C-18 pretreatment depleted the total protein concentration by 60%. How-

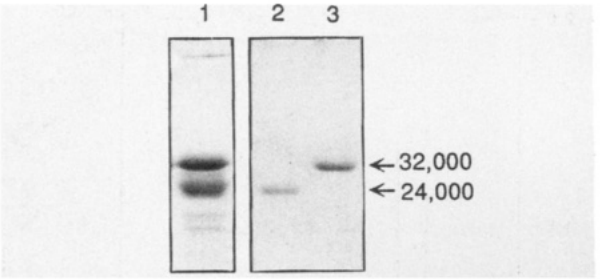

Figure 2. Electrophoretic analysis of purified wine proteins. Proteins were obtained by preparative anion-exchange chromatography as shown in Figure 1. SDS–PAGE of the starting material is shown in lane 1, the fraction eluting at 70 mM NaCl in lane 2, and the fraction eluting at 160 mM NaCl in lane 3. Staining to detect protein was achieved with Coomassie Brilliant Blue and then the more sensitive silver stain procedure to detect impurities. For details see Materials and Methods.

Table I. Purification of Wine Proteins That Contribute to Haze

purifn step	fraction	mg/L of wine[a]	% yield
1	70% $(NH_4)_2SO_4$ ppt	99.5	100
2	C-18 treated sample	40.2	40
3	M_r 24 000 protein	7.9	8
3	M_r 32 000 protein	6.4	6

[a] Protein concentration, calculated from the sum of the masses of amino acids present after acid hydrolysis of the protein fractions, is expressed as milligrams of protein per liter of wine from which all fractions were obtained.

Table II. Amino Acid Composition of Wine Proteins That Contribute to Haze

	amino acid composition, % molar concn			
amino acid	70% $(NH_4)_2SO_4$ ppt	C-18 treated sample	M_r 24 000 protein	M_r 32 000 protein
Gly	13.21	13.36	13.20	15.32
Ala	8.27	8.68	8.43	9.76
Val	5.06	4.06	3.94	3.77
Ile	3.49	3.51	3.24	3.80
Leu	4.80	4.82	4.86	4.39
Ser	8.30	8.59	7.77	9.99
Thr	11.00	9.99	11.19	7.60
Phe	7.06	7.16	7.93	6.64
Tyr	6.45	6.48	6.01	8.21
Trp	0.47	0.61	0.83	0.15
Lys	4.17	4.31	4.49	3.06
Arg	3.99	3.89	4.30	3.20
His	1.94	2.28	1.83	2.58
Asx	15.29	15.63	15.67	14.58
Glx	6.51	6.62	6.31	6.97

ever, the protein composition of the sample after C-18 treatment was not altered as evidenced by the electrophoretic profile (data not shown) and the amino acid composition (Table II). These results suggest that the C-18 removed an equal proportion of all proteins present, possibly as a result of their association with polyphenolic compounds. The two purified proteins totaled 14% of the starting material or 35% of that loaded onto the column.

(*b*) *Amino Acid Composition.* Table II gives the amino acid compositions observed for the M_r 24 000 and 32 000 proteins as well as for the starting material [70% $(NH_4)_2SO_4$ ppt] before and after C-18 treatment. As noted previously (Waters et al., 1991), proline was not detected with the precolumn derivatization technique employed. The amino acid compositions of the 70% $(NH_4)_2SO_4$ ppt and of the C-18 treated sample were almost identical, indicating that the protein composition was unaltered by passage through the reversed-phase adsorbent as discussed

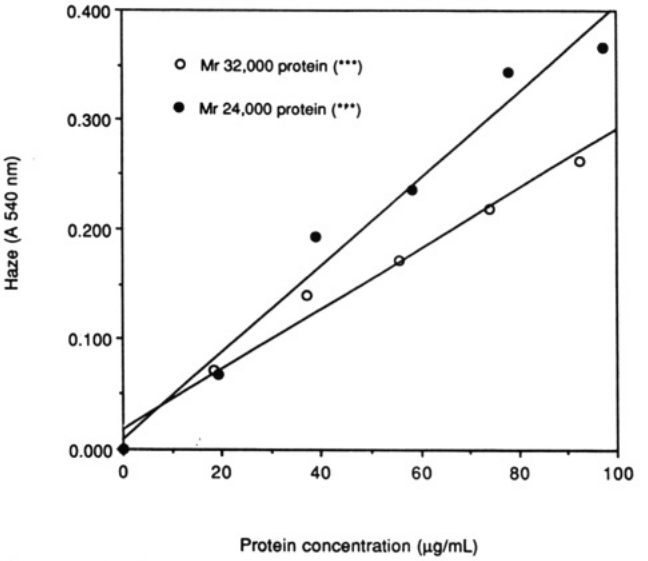

Figure 3. Influence of concentration of purified wine proteins on heat-induced haze. The M_r 24 000 protein and M_r 32 000 protein were back-added to protein-free wine and heated at 80 °C for 6 h followed by 4 °C for 16 h. Haze was measured by A_{540nm}, and protein concentration was determined from the sum of the masses of amino acids present after acid hydrolysis of the proteins as described in Waters et al. (1991). (***) Significant linear correlation at 0.001% level.

above. Both samples contained a large proportion of either aspartic acid or asparagine (Asx), and since it is known that wine proteins are acidic (Hsu and Heatherbell, 1987a; Correa et al., 1988; Moio and Addeo, 1989), it is assumed that Asx was dominated by aspartic acid. The samples were also rich in the aliphatic amino acids glycine, alanine, serine, and threonine and the aromatic amino acids phenylalanine and tyrosine.

The amino acid compositions of the two purified proteins were distinct from each other. The main difference was in their serine and threonine contents: the M_r 32 000 had more serine and the M_r 24 000 more threonine. There were also differences in aromatic and basic amino acids. The M_r 24 000 protein had more phenylalanine and more of the basic amino acids lysine and arginine.

(*c*) *Haze Potential.* The haze potential of the two purified proteins was established by measuring the amount of haze induced by heating a known concentration of each protein in wine (Figure 3). The M_r 24 000 protein caused approximately 50% more haze, at the same concentration, than the M_r 32 000 protein, demonstrating that the M_r 24 000 protein was more important to wine heat haze than the M_r 32 000 protein. The haze yields for the purified proteins were about 2 times higher than those seen with the crude fractions. This further confirms that these two proteins are the most important to haze because they had a higher haze potential than other wine proteins present in the Muscat of Alexandria wine studied.

The identification of this protein as the most susceptible to heat-induced haze has been independently supported by a recent paper which showed that a juice fraction containing M_r 21 000 and 23 000 proteins was important to haze (Paetzold et al., 1990). The results also confirm other papers in the literature (Moretti and Berg, 1965; Mesrob et al., 1983; Hsu and Heatherbell, 1987b), which suggested that grape and wine proteins of low p*I* are important to wine haze, because both of the proteins isolated in this study possessed sufficient negative charge at pH 8.0 to be retained by the anion-exchange column.

Proteolytic Susceptibility of Proteins in Wine. (*a*) *Activity of the Peptidase Preparation on BSA in Wine.*

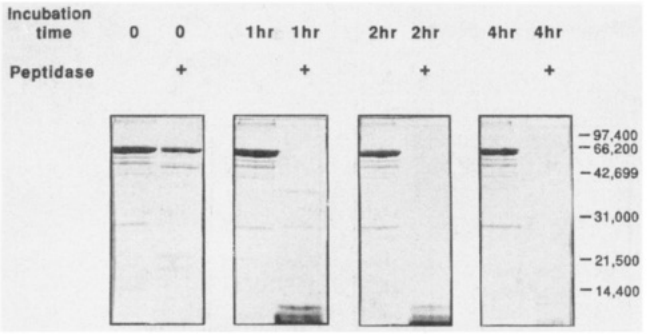

Figure 4. Peptidase susceptibility of BSA in ultrafiltered wine. SDS–PAGE analysis of BSA in ultrafiltered wine incubated at 15 °C with (+) or without the peptidase preparation, Vinozym P, for 0, 1, 2, and 4 h. The positions of the M_r markers are shown on the right side of the gel.

The activity of the peptidase preparation in wine was checked using bovine serum albumin (BSA) as a substrate. BSA was a suitable model for this experiment because it has a haze potential similar to that of wine proteins (Waters et al., 1991). The peptidase preparation, Vinozym P, was chosen because it had previously demonstrated good activity on model substrates in wine (Waters et al., 1990) and was a mixture of mechanistically different peptidases, as demonstrated by its partial inhibition by a range of different active site inhibitors (Waters, 1991).

BSA was added to ultrafiltered, i.e., protein-free, wine treated with Vinozym P over 7 days at 15 °C, and the effects were monitored by SDS–PAGE analysis (Figure 4). The blank samples with no peptidase added showed typical profiles for commercially available BSA; i.e., they were dominated by the BSA band at M_r 66 200 and contained other lower M_r bands presumably corresponding to impurities. The blank samples also showed no evidence of degradation even after 7 days (not shown), indicating that neither the ultrafiltered wine nor the BSA preparation itself had any proteolytic activity. In contrast, the enzyme-treated samples showed evidence of very rapid degradation. Even the time zero sample (i.e., less than 10 s of incubation with the enzyme) showed a decreased amount of the BSA band, the appearance of lower M_r bands, and the loss of the impurity bands, indicating that some hydrolysis had occurred in this short time period. The degradation continued over the next 2 h and was complete by 4 h. Proteins from the peptidase preparation did not interfere with the analysis because the preparation did not contribute observable bands at the concentration at which it was used in these experiments.

These results demonstrate the high activity shown by the peptidase preparation in wine even at this low temperature. The results also confirm earlier data showing that a number of peptidase preparations, including Vinozym P, were active in wine on other proteins such as collagen and casein (Waters et al., 1990; Waters, 1991). The particularly high susceptibility of BSA to proteolysis may be a result of its conformation into three domains allowing the peptidases to hydrolyze peptide bonds in the exposed linking regions, this being the trigger for its general hydrolysis (Peters, 1985).

(*b*) *Peptidase Susceptibility of the Purified 24 000 M_r Wine Protein.* The M_r 24 000 wine protein was back-added to ultrafiltered wine and treated with Vinozym P in the same manner as BSA had been treated in the control experiment. The results of SDS–PAGE analyses of samples taken at intervals during the course of the experiment are shown in Figure 5. Not all samples were run on the same gel. Although efforts were made to stain the gels to similar intensities, some pairs are stained darker than others. However, comparison between incubation times is not as important as comparisons of the control to the enzyme-treated sample within the same incubation time. All such pairs show identical staining because they were run on the same gel.

The M_r 24 000 wine protein showed no degradation even after 2 weeks of incubation with the enzyme. This is in contrast to BSA in the control experiment, which had been completely hydrolyzed within 4 h. Demonstration of the resistance of the M_r 24 000 protein to proteolysis eliminates the possibility, at least for this protein, that wine polysaccharides act as protective colloids because such polysaccharides were removed in the preparation of the wine medium and the 24 000 M_r protein substrate. However, the wine protein itself may be a peptidase inhibitor, thus preventing its own hydrolysis. To assess this possibility, BSA was treated with the peptidase preparation in the presence of this putative inhibitor.

(*c*) *Effect of Wine Colloids on the Peptidase Susceptibility of BSA.* BSA was added to Muscat of Alexandria wine containing the full complement of wine macromolecules (including the M_r 24 000 protein examined above) and treated with Vinozym P. The results of SDS–PAGE analysis are shown in Figure 6. In the time zero sample, bands contributed by the added BSA in addition to fainter bands in the M_r range 34 000–24 000 corresponding to the wine proteins were observed. As occurred in the control experiment in ultrafiltered wine (Figure 4), BSA was rapidly hydrolyzed and showed complete degradation within 4 h. Nevertheless, the wine proteins remained unaffected by the peptidase activity of Vinozym P. Data for the 2- and 9-day incubations (Figure 6), which were obtained with heavily stained gels, confirm the resistance of the wine proteins, including the M_r 32 000 protein, to proteolysis with Vinozym P. After 9 days of incubation in wine, the blank sample also showed some evidence of BSA degradation, indicating that the wine contained some natural peptidase activity.

The rapid degradation of BSA in the presence of wine macromolecules discounts the hypothesis that natural wine proteins or other wine components are inhibitors of peptidase activity. This result also shows that the wine polysaccharides did not act as protective colloids.

CONCLUSION

The purification of two of the major wine proteins to homogeneity has led to the positive identification of proteins that are involved in wine instability, and their removal is therefore critical for white wine stabilization to be achieved. The identification of the M_r 24 000 protein as the most important to heat hazing has also provided a substrate for the evaluation of alternative protein removal techniques such as peptidase treatment.

The peptidase preparation used in this study contained a range of different peptidases (Waters, 1991) and showed rapid activity in wine on BSA. The degradation of BSA in wine was not affected by the presence of wine polysaccharides, and hydrolysis proceeded as rapidly in wine containing all of the wine colloids as it did in ultrafiltered wine which was free of any macromolecules greater than 10 000 M_r. This demonstrates that wine does not contain low or high M_r peptidase inhibitors.

Wine proteins showed a remarkable resistance to this highly active peptidase preparation, and this resistance was shown in the presence or absence of other wine macromolecules such as polysaccharides. This indicates that the resistance of native wine proteins to proteolysis is an inherent property of these molecules. Indeed, if one

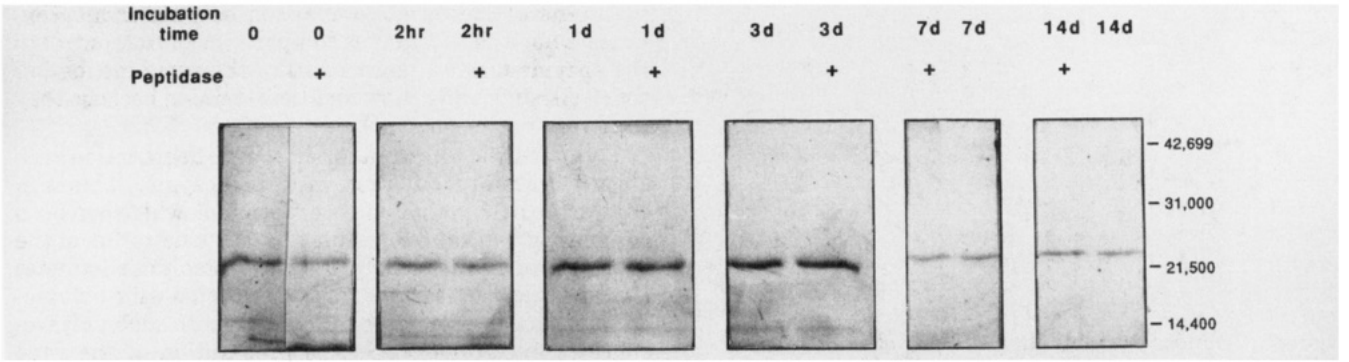

Figure 5. Peptidase susceptibility of M_r 24 000 wine protein in ultrafiltered wine. SDS–PAGE analysis of the M_r 24 000 wine protein in ultrafiltered wine incubated at 15 °C with (+) or without the peptidase preparation, Vinozym P, for 0 and 2 h and 1, 3, 7, and 14 days. The positions of the M_r markers are shown on the right side of the gel. (Note that the peptidase treatment is shown in the left lane of the gels at 7 and 14 days.)

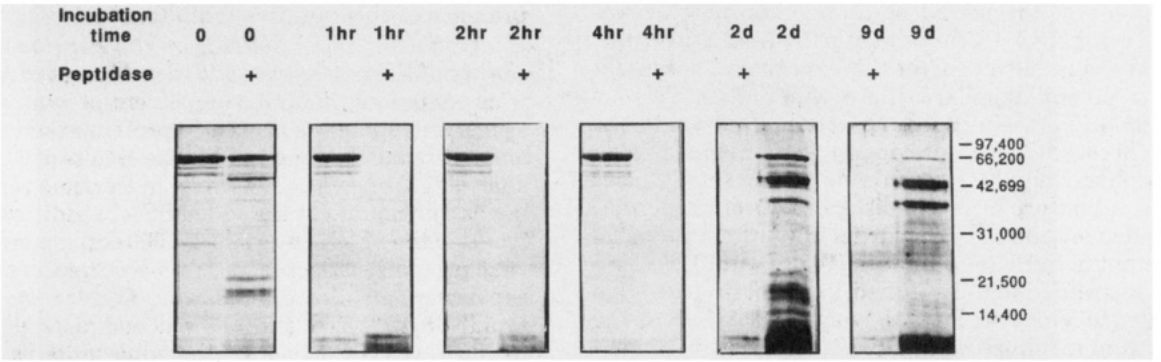

Figure 6. Peptidase susceptibility of BSA in wine containing wine macromolecules. SDS–PAGE analysis of BSA in wine incubated at 15 °C with (+) or without the peptidase preparation, Vinozym P, for 0, 1, 2, and 4 h and 2 and 9 days. The positions of the M_r markers are shown on the right side of the gel. (Note that the peptidase treatment is shown in the left lane of the gels at 2 and 9 days.)

considers that these proteins have survived the winemaking process and that natural grape peptidases are present in the wine, it is unsurprising to find them resistant to proteolytic attack.

Resistance to proteolysis is not unique to these grape and wine proteins, and numerous examples of other resistant proteins exist in the literature (North, 1989). Glycosylation can sometimes confer protection to proteins (Semino et al., 1985; Maruyama et al., 1990; Seymour et al., 1991), but there are conflicting reports in the literature about the glycosylation status of wine proteins. Paetzold et al. (1990) and Yokotsuka et al. (1991) claimed that all grape and wine proteins were glycosylated. In contrast, previous studies with the protein fractions described here detected only one wine glycoprotein with M_r 34 000 (Waters, 1991), and Hsu and Heatherbell (1987a) detected only three glycoproteins with M_rs of 12 600, 25 000, and 28 000 in other juices and wines, suggesting that the occurrence of glycosylated proteins in wine may not be common. However, the extent of glycosylation of any particular wine protein is unknown.

Complexation of the wine proteins with phenolic compounds has also been suggested as a reason for their resistance to proteolytic hydrolysis (Heatherbell et al., 1984), and although this property of wine proteins was not examined in this study, it seems unlikely to be the cause of the resistance observed. This is because BSA, which is known to bind phenolic (Throneberry, 1961; Weinbach and Garbus, 1966; Hobson, 1970), was not protected from hydrolysis when present in a medium containing phenolics (i.e., ultrafiltered wine). Nevertheless, the role of phenolic compounds in the enzymic degradation of wine proteins, and in other aspects of protein stability, should be examined further.

This study adds further support to the growing body of evidence (Ngaba-Mbiakop, 1981; Feuillat and Ferrari, 1982; Heatherbell et al., 1984; Modra and Williams, 1988; Waters et al., 1990) that treatment of juices and wines with proteolytic enzymes at the low temperatures appropriate to winemaking will not confer protection against protein precipitation. This is because the wine proteins which show the highest susceptibility to heat-induced haze are not hydrolyzed under these conditions.

ABBREVIATIONS USED

Asx, aspartic acid or asparagine (undefined); BSA, bovine serum albumin; Glx, glutamic acid or glutamine (undefined); M_r, relative molecular mass; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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