Saccharomyces cerevisiae Mannoproteins That Protect Wine from Protein Haze: Their Release during Fermentation and Lees Contact and a Proposal for Their Mechanism of Action

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A fraction containing the mannoproteins released during fermentation from the winemaking strain of *Saccharomyces cerevisiae*, Maurivin PDM, was able to reduce the visible protein haze in white wine. This fraction of haze protective mannoprotein material (HPM) could be recovered by either ultrafiltration or ethanol precipitation. The kinetics of the release of both mannose- and glucose-containing polymers during the growth cycle of PDM were determined as a guide to the release of HPM. Active HPM was first detected in the culture supernatant when the cells were exponentially growing. HPM was also released into the medium under an environment simulating winemaking conditions by PDM cells during fermentation as well as during storage on yeast lees. Since the amounts of HPM released during fermentation are greater than those subsequently extracted from the cell wall, fermentation would be a more viable procedure than extraction from yeast cells for the commercial production of HPM. Yeast invertase, a mannoprotein with haze protective activity, was used as a model substrate to investigate the mechanism of haze protection. Invertase was found to reduce visible turbidity but not prevent protein precipitation. Invertase itself did not precipitate but remained soluble in the wine. On the basis of these observations, we propose that the mechanism of haze protection may be one of competition between HPM and wine proteins for unknown wine component(s), the latter being required for the formation of large insoluble aggregates of denatured protein. As the available concentration of these components decreases, due to the presence of HPM, the particle size of the haze decreases and thus visible turbidity declines.

Keywords: *Yeast cell wall; mannoprotein release; wine protein haze; haze protective material; autolysis; fermentation*

INTRODUCTION

White wine clarity is of prime importance for the winemaker as a bottle showing haziness is likely to be rejected by the consumer. The most common form of haze formation in white wine results from the aggregation of grape proteins naturally present in wine (Paetzold et al., 1990; Waters et al., 1991). To prevent haze formation, winemakers usually lower the concentration of wine proteins through the use of bentonite. Bentonite also results in the costly loss of wine in lees and removes wine aroma components, hence lowering wine quality (Miller et al., 1985; Puidgeu et al., 1996). Thus, alternative methods of protein stabilization are being investigated by the wine industry.

We have isolated two high-M_r mannoproteins from fermentations by a winemaking strain of *Saccharomyces cerevisiae* of white or red grape juice (HPF1, Waters et al., 1993; Waters et al., 1994a) or of chemically defined grape juice medium (HPF2, Stockdale et al., unpublished) that are able to prevent visible wine protein haze formation. Putative structural genes for both mannoproteins have been identified in the *S. cerevisiae* genome (Waters, unpublished; Stockdale et al., unpublished). Mannoprotein material with haze protective activity (i.e. haze protective mannoprotein material, HPM) could also be extracted from cells or cell walls of winemaking strains of *S. cerevisiae* (Dupin, 1997; Dupin et al., 2000).

Independently, Ledoux et al. (1992) showed that wine aged on yeast lees had lower haze potential and lower bentonite requirements for stability than wine aged without lees but containing the same level of protein. The active component was identified as a 32 kDa fragment of yeast invertase, a yeast periplasmic enzyme, and could be enzymatically extracted from yeast cell wall preparations (Moine-Ledoux and Dubourdieu, 1999).

Other glycoproteins have also been shown to exhibit haze protective activity. These include whole yeast invertase (McKinnon, 1996; Moine-Ledoux and Dubourdieu, 1999), a wine arabinogalactan−protein (Waters et al., 1994b), gum arabic, and an apple arabinogalactan−protein (Pellerin et al., 1994). All of these active glycoproteins have a relatively high proportion of carbohydrate to protein. The importance of the carbohydrate

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10.1021/jf0002443 CCC: $19.00 © 2000 American Chemical Society
Published on Web 07/22/2000

moiety of the wine arabinogalactan–protein (AGP) with haze protective activity was studied (Waters et al., 1994b). Enzymatic removal of the terminal arabinofuranosyl residues of the AGP and subsequent partial shortening of the outer 6-linked galactan chains did not effect the haze protective activity. Periodate oxidation and then Smith degradation of the AGP eliminated its haze protective activity but also greatly reduced the amount of protein. It also removed the majority of the outer 6-linked galactan chains. It was thus speculated that the outer 6-linked galactan chains and/or the protein portion of the AGP was important for haze protective activity.

The precise mechanism for haze protection of heat unstable proteins remains unclear. It has, however, been established that addition of haze protective mannoproteins did not prevent the proteins in wine from precipitating but decreased the particle size of the haze (Waters et al., 1993). An unpurified yeast mannoprotein fraction isolated from wine decreased wine haze particle size to 5 μm, and the haze was barely detectable with the naked eye (Waters et al., 1993).

Clearly, the use of HPM as a replacement for bentonite fining is an exciting prospect for the wine industry to help alleviate protein instability problems. A process to obtain the active 32 kDa fragment of invertase discussed above by enzymatic digestion of yeast cell walls has been described (Moine-Ledoux and Dubourdieu, 1999). We have also shown that HPM could be extracted from cells or cell walls of various winemaking strains of *S. cerevisiae* (Dupin, 1997; Dupin et al., 2000). The yields of HPM extracted in these studies was low, and this means that industrial production of HPM by extracting cells or cell walls is probably not realistic. It is relevant to note that HPM was first isolated from wine (Waters et al., 1993; Waters et al., 1994a) rather than the cells themselves. Some HPM may have been released from the cell wall during yeast cell degeneration at the end of fermentation of these wines, but since the postfermentation time was short, it is possible that HPM was secreted during fermentation.

Mannoproteins from yeast cell walls are known to be released into the extracellular medium during yeast growth and autolysis (Llaubères et al., 1987; Sijmons et al., 1987; Feuillat et al., 1989; Charpentier and Feuillat, 1993), and their release is generally considered beneficial. Mannoproteins can interact with aroma compounds and thus potentially change the sensory properties of wine (Lubbers et al., 1994a; Lubbers et al., 1994b; Lavigne and Dubourdieu, 1996; Dufour and Bayonove, 1999). Their release can also stimulate the growth of lactic acid bacteria and thus aid in the timely completion of malolactic fermentation (Guilloux-Benatier et al., 1995). As well as potentially protecting wine from protein haze, which is the subject of this current work, mannoproteins can also protect wine from tartaric acid precipitation (Lubbers et al., 1993, Gerbaud et al., 1997; Moine-Ledoux et al., 1997).

In this paper, we have examined the release of mannoproteins by a commonly used winemaking strain of *S. cerevisiae*, Maurivin PDM, into the extracellular medium at different phases of yeast growth and assessed any such release as a possible source HPM. In addition, the mechanism of haze protection by such mannoproteins has been examined using yeast invertase as a model.

MATERIALS AND METHODS

Strains, Medium, and Propagation Conditions. *Saccharomyces cerevisiae* Maurivin PDM was obtained from Mauri Foods yeast group (Sydney, Australia). Yeast maintained on a yeast peptone dextrose slope was propagated in chemically defined grape juice medium (CDGJM) containing either glucose (200 g/L) or mannose (150 g/L), as described in Dupin et al. (2000). The details of further propagation steps are given below. Yeast growth was monitored by measuring the absorbance at 650 nm of the fermentation culture. The morphology of the cells, including budding, was also assessed by phase contrast microscopy.

Small-Scale (10 L) Fermentation Trials Conducted at 25 °C with Agitation. Four propagation steps into CDGJM containing either glucose (200 g/L) or mannose (150 g/L) were undertaken, with culture volumes at each step being 3, 25, 500, and 9500 mL. Cells were grown at 25 °C with agitation to late exponential phase (7.5–11.5 g/L, wet cell weight, approximately 1.2×10^8 cfu/L) or stationary phase (16 g/L, wet cell weight, approximately 1.9×10^8 cfu/L). The culture supernatant from each fermentation experiment was recovered by centrifugation (18 000*g*, 10 min, 5 °C), filtered (0.45 μm membrane), and stored at −20 °C. The culture supernatants were ultrafiltered (YM 10 membrane, 10 kDa molecular weight cutoff, Amicon Ltd., Danvers, MA). The retentate was collected, freeze-dried and weighed.

Alternatively, the culture supernatant was mixed with 96% (v/v) ethanol (3 volumes) and left at −20 °C for 48 h. The mannoprotein precipitate was recovered by centrifugation (18 000*g*, 15 min, −10 °C) and washed with 75% (v/v) ethanol before being dissolved in water, freeze-dried, and weighed.

Affinity chromatography on Concanavalin A of the ultrafiltered material was carried out as described in Dupin et al. (2000).

Large-Scale (20 L) Fermentation Conducted at 25 °C with Agitation. Four propagation steps into CDGJM containing glucose (200 g/L) were undertaken, with culture volumes at each step being 10 mL, 50 mL, 1 L, and 19 L. Final growth of yeast was carried out in a 28 L vessel at 25 °C with agitation on an orbital shaker (110 rpm, Paton Scientific, model OP 3422, Adelaide, Australia). The following volume of culture (in parentheses) was sampled under nitrogen gas cover at the following times: 0 h (1.5 L); 3, 6, 8, 10, and 13 h (all 1 L); 17 h (750 mL); 20 and 23 h (both 500 mL); 28, 31, 34, and 37 h (all 300 mL); 41, 50, 60, 83, 104, 126, and 150 h (all 250 mL) after inoculation. The culture supernatant was recovered from the samples by centrifugation (18 000*g*, 10 min, 5 °C), filtered (0.45 μm membrane), and stored at −20 °C. The culture supernatants (175 mL) were dialyzed against distilled water (10 L, changed four times), freeze-dried, and weighed.

Large-Scale (15 L) Fermentation at 18 °C without Agitation and Storage on Yeast Lees at 18 °C. Four propagation steps into CDGJM containing glucose (200 g/L) were undertaken, with culture volumes at each step being 10 mL, 40 mL, 750 mL, and 14.25 L. Final growth of yeast was carried out in a 20 L vessel at 18 °C without agitation.

The culture was sampled daily (1 L, after gentle resuspension of the cells) under nitrogen gas cover, and the culture supernatant was recovered by centrifugation (4000*g*, 10 min, 10 °C), filtered (0.45 μm membrane), and stored at −20 °C. The culture supernatants were ultrafiltered (Amicon YM 10 membrane). The retentate was collected, freeze-dried, and weighed.

At the completion of the fermentation, yeast cells were separated from the culture supernatant by centrifugation (4000*g*, 10 min, 10 °C). The supernatant was ultrafiltered (YM 30 membrane, 30 kDa molecular weight cutoff, Amicon Ltd.) while the yeast pellet was resuspended in culture (200 mL) at 4 °C. The retentate from the ultrafiltration contained the extracellular mannoprotein material released into the culture during fermentation and was kept for further analyses. The ultrafiltered medium (mannoprotein-free medium, 10 L) was sterile filtered and transferred into a sterile, airtight storage vessel (15 L). The yeast cells were separated from the culture

supernatant by centrifugation (4000*g*, 10 min, 10 °C), and the pellet was back added to the mannoprotein-free medium under aseptic conditions. The air in the headspace of the storage vessel was replaced with nitrogen gas, and the vessel was sealed and stored at 18 °C.

Sampling (1 L, after gentle resuspension of yeast lees) was performed every 2 weeks for a 2-month period under nitrogen gas cover. These samples were centrifuged (4000*g*, 10 min, 10 °C), and the recovered supernatants were stored at −20 °C before use. After being thawed, the samples were ultrafiltered (Amicon YM 10 membrane) and the retentate was collected, freeze-dried, and weighed.

Determination of Total and Viable Cell Counts. The total cell density in the culture was determined using a Neubauer counting chamber (minimum of 600 cells counted when possible, accuracy 99%). The viable cell density was determined using the spread plate counting method. The number of viable cells, expressed in colony-forming units (CFU), corresponded to the number of colonies counted after 24 h of incubation at 25 °C. For each time point, all cell counts were done in triplicate.

Determination of Polymeric Mannose and Glucose Contents. The concentration of mannoproteins in the samples was determined by the enzymatic method developed by Ryan (1998). For samples obtained from fermentations or wines, the samples were desalted on a PD6G column (BioRad Laboratories, Sydney, Australia) into water. Polymeric forms of mannose and glucose present in the samples were hydrolyzed into monomeric sugars by addition of sulfuric acid (final concentration 1.5 M) to the sample. The solution was heated for 90 min at 100 °C in sealed glass tubes. Cooled hydrolyzed samples (60 μL) were transferred to microplate wells and neutralized with NaOH (90 μL, 2 M) and triethanolamine buffer (75 μL, 25 mM, pH 7.6). The total amounts of monomeric glucose were determined enzymatically using the D-glucose/D-fructose UV method determination kit (set of enzymes E2 and E3, Roche Diagnostics GmbH, Mannheim, Germany) followed by the determination of the monomeric mannose content with the enzyme phosphomannose isomerase (PMI, Roche Diagnostics).

The absorbance (A_{340} nm) was read prior to the addition of enzymes E2 and E3 (7.5 μL each) and after 1 h of incubation at 25 °C. As yeast cells do not release any fructose or polymers containing fructose (Usseglio-Tomasset, 1978), the enzymes E2 and E3 were used simultaneously and the corresponding A_{340} nm reading after incubation was taken as the measure of the glucose content only. PMI (8 μL) was added and after 1 h at 25 °C the A_{340} nm was again taken. The difference between A_{340} nm before and after PMI addition was a measure of the concentration of mannose in the sample.

The monomeric glucose and mannose content initially present in the samples was measured enzymatically as above with omission of the hydrolysis step. The content of polymeric sugars was calculated by subtraction of the initial monomeric content before hydrolysis from the total content of monomeric sugars after hydrolysis.

Each measurement for the enzymatic assay was repeated at least three times or until an average value with a standard deviation lower than 5% was reached. Known amounts of monomeric D-(+)-mannose and β-D-(+)-glucose were used to determine the standard curves. Values for glucose and mannose concentrations of samples were calculated from the regression equation of the standard curves.

Micromethod for the Measurement of the Heat-Induced Haze (Heat Test). Except for the experiments with yeast invertase, the effects of mannoprotein addition on the protein haze potential were determined as described in Dupin et al. (2000).

For yeast invertase (Sigma Chemical Co., St. Louis, MO), the method described in Dupin et al. (2000) was modified as follows. Wine was commercially produced from *Vitis vinifera* L. Sauvignon Blanc grapes. The protein concentration of the wine was estimated to be 240 mg/L by the method of Peng et al. (1997). Aqueous solutions of invertase (0−200 μL, made up to 200 μL with water, final concentration of 0, 100, 200, 300, or 400 mg/L on dry weight basis) were added to the wine

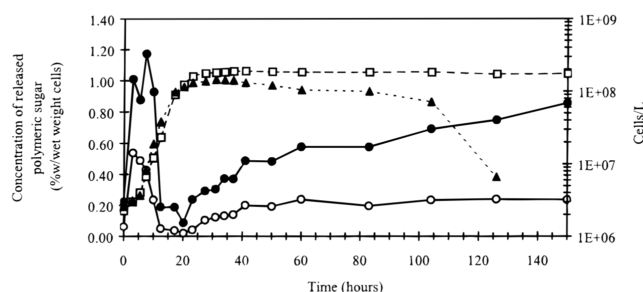

Figure 1. Change in the concentration of polymeric mannose (●) and polymeric glucose (○) in the culture supernatant during growth of PDM cells. Both the total cells (□) and viable cells (▲) are shown. The values are the mean of at least three analyses.

(10 mL). After being mixed and sealed, the samples were heated for 6 h at 80 °C and left overnight at 4 °C. After 20 min at room temperature, the samples were mixed to disperse the haze and an aliquot of each sample (100 μL) was transferred to a 96 well flat-bottomed microplate. The turbidity was measured by the absorbance of the samples at 490 nm on a UV max microplate reader (Molecular Device Corp, Hopkinton, MA). Values were corrected by subtraction of the absorbance at 490 nm for a control (unheated wine).

SDS PAGE Analysis of Hazes. The haze from 1 mL of wine was isolated by centrifugation (10 000*g*, 5 min), SDS PAGE sample buffer was added (30 μL, Laemmli, 1970), and the haze was resuspended with a vortex and boiled for 10 min. After cooling, the samples were centrifuged (10 000*g*, 5 min), and the supernatant was loaded onto a 12% denaturing polyacrylamide gel. SDS PAGE analysis was performed as described by Laemmli (1970).

Protein Quantification by HPLC Analysis. The protein composition of heated and unheated wines was determined using the reversed phase HPLC method described by Peng et al. (1997).

RESULTS

Release of HPM by PDM Cells during Fermentation at 25 °C. The release of polymers containing mannose and glucose ("polymeric mannose" and "polymeric glucose") by yeast during fermentation was examined (Figure 1). Fermentation was carried out on a relatively large scale (20 L) in a chemically defined grape juice medium at 25 °C, and the culture was continuously agitated. During the adaptation phase of growth (from zero to approximately 6 h after inoculation, Figure 1) and in the first hours of exponential growth (from 6 to 8 h), the quantity of polymeric mannose as well as polymeric glucose released into the culture fluid per gram of biomass dramatically increased. Analyses of samples from mid to late exponential phase (from 8 to 17 h) revealed a subsequent decrease in content of polymeric mannose and glucose. The content of polymeric mannose in the culture supernatant increased again during the transition from exponential to stationary phase (from 18 to 30 h), during stationary phase (30 to 83 h), and during decline phase (more than 83 h). The release of polymeric glucose was concomitant with the release of polymeric mannose and seemed to be partially correlated to it as the ratio of polymeric mannose to glucose was relatively constant (ranged from 2.4 to 2.8) over transition and early stationary phase (from 28 to 60 h of incubation).

A change occurred during decline phase (more than 83 h of incubation, Figure 1) as the ratio of polymeric mannose to glucose steadily increased to reach 3.6. The amount of polymeric glucose did not decrease over this

Table 1. Yield and Haze Protective Activity of the Extracellular Material Released into the Culture Medium by *Saccharomyces cerevisiae* Maurivin PDM Cells at Different Stages of Growth at 25 °C with Agitation[a]

carbohydr source	yield of released mater (% w/w)[b]		haze protective activity of released mater (% haze)[c]	
	LEP	SP	LEP	SP
glucose	0.67	1.07 (0.74)	30	35 (25)
mannose	0.83	1.58	26	23

[a] LEP, late exponential phase; SP, stationary phase. [b] Results are expressed as % (w/w) of extracellular material released (dry weight) per wet weight of yeast cells. Samples were prepared from culture supernatants by ultrafiltration except for the values given in parentheses, which were prepared by ethanol precipitation. The values given are the means of at least four independent experiments except for the data from ethanol precipitation and from growth on mannose, which are from a single experiment. [c] Extent of haze decrease as a percentage of the initial haze value (as observed with no extracellular material added) seen at a concentration of 0.5 mg of extracellular material/mL wine in the micromethod for the measurement of the heat-induced haze (values are the means of three independent experiments).

period (values varied between 27 and 33 mg/L) whereas the amount of polymeric mannose increased from 80 to 102 mg/L. As seen in Figure 1, the number of viable cells decreased over this period.

An additional experiment was carried out to examine the haze protective activity of material released during fermentation. Yeast was grown under conditions identical to those of the experiment described above except the scale was smaller (10 L). The haze protective activity of the secreted material was tested by comparing the level of haze produced from heating the protein BSA in wine with and without the secreted material. Previous studies have shown that BSA reacts similarly to wine proteins under these conditions (Waters et al., 1993). Due to the variability of the heat test (up to than 10% standard deviation), the activity of the tested samples has been classified into four broad categories. First, at concentrations of secreted material of up to 0.5 g/L, material classified as having "above average", and "average" activity reduced the control haze value (the amount of haze given by BSA alone) to between 20% and 40% and between 40% and 60%, respectively. In addition, as the concentration of above average or average samples increased beyond 0.5 g/L, the percentage of haze either decreased further or remained constant. Secreted material classified as having "borderline" activity reduced the haze to between 60% and 90% of the control haze value at a concentration of secreted material of 0.5 g/L, but the percentage of haze tended to increase with higher concentrations. Material classified as having no activity gave haze values that were greater than 90% of the control haze value.

All extracellular material tested showed above average haze protective activity (Table 1). This result was seen whether the material was isolated from culture supernatant at the late exponential or the stationary phase of yeast growth, whether the yeast was grown on mannose or glucose as the carbon source, and whether ethanol precipitation or ultrafiltration was used to isolate the material.

Consistent with the trends shown in Figure 1, lower yields of released material were obtained from culture supernatants at the late exponential phase than at the stationary phase of yeast growth (Table 1). Yields at both growth phases were slightly higher if mannose was used instead of glucose as the carbon source (Table 1). The yield of extracellular material from culture supernatants at the stationary phase of yeast growth isolated by ethanol precipitation was 30% lower than that obtained by ultrafiltration (Table 1, values in parentheses). The yields of secreted material determined in this experiment (Table 1) are not directly comparable to those in the experiment described above (Figure 1) since dry weight of total material was measured in Table 1 whereas levels of polymeric mannose and glucose containing polymers were determined in the experiment described in Figure 1.

Table 2. Concentration of Polymeric Mannose in the Culture Supernatant and Haze Protective Activity of the Extracellular Material Released by *Saccharomyces cerevisiae* Maurivin PDM Cells during Fermentation under Conditions Simulating Winemaking[a]

yeast cell growth stage	concn of released polymeric mannose (mg/L)	haze protective activity of released mater (% haze)[b]
mid exponential	nd	80
late exponential	104	40
mid stationary	175	30

[a] nd, not determined. [b] Extent of haze decrease as a percentage of the initial haze value (as observed with no extracellular material added) seen at a concentration of 0.5 mg of extracellular material/mL of wine in the micromethod for the measurement of the heat-induced haze.

Release of Polymeric Mannose and HPM during Fermentation Conditions Simulating Winemaking (Nonagitated at 18 °C). A fermentation in chemically defined grape juice medium was conducted under conditions more closely simulating winemaking than those conditions described above. Sampling was performed regularly during the exponential growth of the yeast cells and the following stationary phase of growth. The amount of mannose present in polymeric form released into the culture was determined for selected samples only and is given in Table 2.

As seen previously for the agitated fermentations at 25 °C, the transition between exponential and stationary phase under winemaking conditions was characterized by an increase of polymeric mannose present in the culture supernatant.

The extracellular material collected at mid exponential phase showed only borderline haze protective activity (Table 2) suggesting that HPM was either absent or present in an amount too low to be detected. However, tests on samples at late exponential or stationary phase revealed the presence of HPM because these samples showed above average activity (Table 2). As described above for the agitated fermentations at 25 °C, it appears that HPM was released during cell growth.

Release of Polymeric Mannose and HPM by PDM Cells during Storage on Yeast Lees. To measure the effects of storage on yeast lees under winemaking conditions, cells were harvested after the nonagitated fermentation at 18 °C described above and resuspended in a medium free of mannoproteins and other polymeric material. The amounts of polymeric mannose measured and the haze protective activity observed were thus related to the extracellular material released exclusively during storage on yeast lees (Table 3).

Polymeric mannose was progressively released into the culture supernatant to reach a concentration during

Table 3. Concentration of Polymeric Mannose and Haze Protective Activity of the Extracellular Material Released into the Medium during Storage on PDM Yeast Lees

time on yeast lees (weeks)	concn of released polymeric mannose (mg/L)	haze protective activity of released material (% haze)[a]
0	0	100
2	4	90
4	10	80
6	11	70
8	15	50

[a] Extent of haze decrease as a percentage of the initial haze value (as observed with no released extracellular material added) seen at a concentration of 0.5 mg of extracellular material/mL of wine in the micromethod for the measurement of the heat-induced haze.

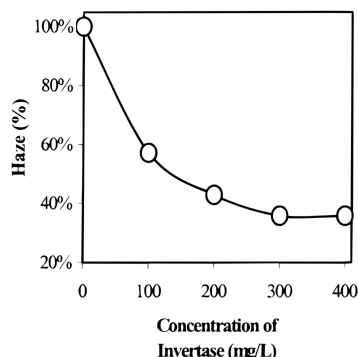

Figure 2. Effect of increasing addition of invertase on wine protein haze, measured as A_{490} and expressed as a percentage of the initial haze value (as observed with no invertase added). The values are the means of 4 analyses.

storage on yeast lees of 15 mg/L after 8 weeks. In parallel, the haze protective activity of the released material increased over time. The first samples tested (from 0 to 6 weeks) showed either no or borderline activity. By 8 weeks, the haze protective activity of the released material had improved to an average level.

Mechanism of Haze Protective Activity of Invertase. The haze protective activity of invertase increased as its concentration in wine increased (Figure 2). In these experiments, invertase was added to a wine containing its natural complement of wine proteins (240 mg/L) rather than to an ultrafiltered wine supplemented with BSA (125 mg/L) as used in all the experiments described above.

Analysis of the haze produced during the heat test by SDS PAGE showed that there was no obvious difference in the type or quantity of proteins among any of the samples (Figure 3). There were 5 major bands with M_r ranging from 12 000 to 35 000 in the haze samples. Invertase was seen in this SDS PAGE analysis as a band with M_r 110 000, but this band did not appear in any of the haze samples. The commercial sample of invertase used in this work also contained a protein band with M_r of 30 000. The presence of invertase in the hazes was also investigated by determining the amount of polymeric mannose present in the hazes and in the unheated and heated wines (Table 4). There was the equivalent of 0−4 mg/L of invertase present in the hazes regardless of the addition rate of invertase. There was a much greater level of polymeric mannose, and by inference, invertase, present in the heated wines (Table 4), and there was a linear correlation between the amount of invertase added and polymeric mannose in the wine after heating ($r^2 = 0.98$).

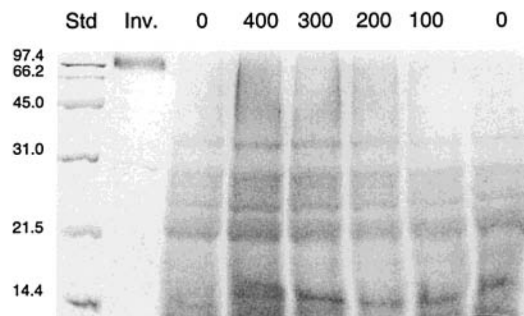

Figure 3. Protein composition as analyzed by SDS PAGE of hazes from 1 mL of wine with no added invertase (0) or with invertase added to the wine at 100−400 μg/mL (100−400). Invertase (100 μg, Inv.) and protein molecular weight standards (Std) were also analyzed. The M_r's of the protein molecular weight standards in kDa are shown on the left of the gel.

Table 4. Effect of Added Invertase on the Concentration of Polymeric Mannose in Unheated and Heated Wines and Hazes from Heated Wines[a]

concn of invertase (mg/L)	concn of polymeric mannose (mg/L) in		
	unheated wine	heated wine	haze[b]
0	110[c]	94	14
100	nd	134 (80)	16 (4)
200	nd	196 (204)	14 (0)
300	nd	246 (304)	12 (0)
400	290 (360)[d]	264 (350)	16 (4)

[a] nd, not determined. [b] The concentration of polymeric mannose in the hazes has been expressed on a mg/L basis by determining the polymeric mannose content of haze isolated from a known volume of wine. [c] Values are means of triplicate analysis. [d] Values in parentheses are the estimated concentration of invertase, calculated by subtracting the concentration of polymeric mannose in the sample with no added invertase and multiplying by 2, since invertase is 50% mannose by weight.

The soluble protein composition of the wines before and after heating was determined by HPLC analysis (Figure 4). The unheated wines without and with added invertase (see Figure 4a,b, respectively) contained identical levels of thaumatin-like proteins (major peak at 11 min, minor peaks at 12−13 min) and chitinases (group of peaks from 18 to 26 min). No protein was detectable with this method for aqueous solutions of invertase (data not shown). After heating, no samples, regardless of invertase addition level, contained soluble protein (Figure 4c).

DISCUSSION

The majority of the material released from yeast during late exponential phase and stationary phase bound to a Con-A column (a lectin which binds mannose and mannose containing polymers; data not shown). This observation confirms reports in the literature (Llaubères et al., 1987; Feuillat et al., 1989) that most material released by yeast growing in grape juice or grape juice like media was mannoprotein in nature. The concentration measured here of secreted material at the end of fermentation (175 mg/L) was also in agreement with the data obtained by others (Llaubères et al., 1987; Feuillat et al., 1989). To utilize mannose in place of glucose as a carbohydrate source appeared to favor the release of more material (Table 1). The increasing yield may be linked to the fact that the mannoprotein content of the cell wall has been shown to be significantly higher when mannose is used as the carbon source instead of glucose (Biely et al., 1971; Krátký et al., 1975).

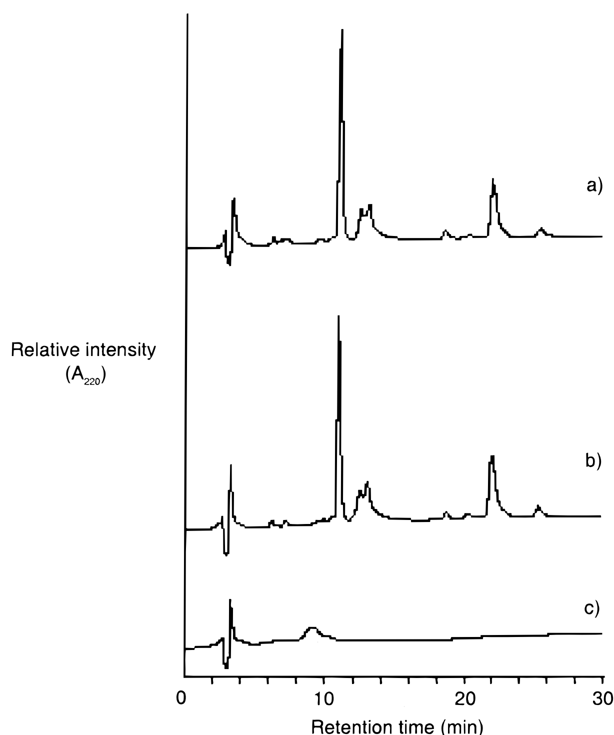

Figure 4. Protein composition of (a) unheated wine with no addition of invertase, (b) unheated wine with added invertase (400 mg/L), and (c) heated wine with added invertase (400 mg/L) as assessed by HPLC analysis.

Kinetics of Release of Mannoproteins from Yeast during Fermentation Complex. Immediately following inoculation, the quantity released into the culture supernatant of polymers containing mannose was very high compared to that at every other phase of cell growth (Figure 1). This large release of mannose containing polymers could be an indirect consequence of the adaptation to osmotic stress (Blomberg and Adler, 1992). In the propagation culture before inoculation, the cells were in a medium depleted in monomeric glucose because a portion of the glucose had been metabolized. When the cells were transferred to the final growth medium where the concentration of monomeric glucose was higher, the cells had to adapt to the new conditions. Cell shrinkage due to the loss of intracellular water might have induced a loss of macromolecules from the cell or cell wall, and this could explain the high release of polymeric sugars observed during the adaptation phase.

During exponential cell growth following this adaptation phase, the concentration of polymeric mannose and glucose in the culture decreased to a lower level than that observed during the adaptation phase. This phenomenon was evident when the data was examined on a per volume basis (data not shown) and can also be seen in Figure 1, where concentration is shown on a biomass basis. A possible explanation for this decrease could be that the polymeric sugars initially released into the culture were degraded into monomers during subsequent growth. It is known that a controlled hydrolysis of the wall occurs during cell budding (exponential phase) since the wall of the mother cells has to be "softened up" locally to allow the emergence of the bud (Fleet, 1991). In addition, the total extracellular β-(1−3)-glucanase activity increases during the budding period and is directly involved in the hydrolysis of the cell wall glucan (Cortat et al., 1972). Hien and Fleet (1983) also showed that *exo*- and *endo*-glucanases were produced during exponential growth of cells.

The content of polymeric mannose in the culture supernatant increased again during the transition from exponential to stationary phase. This observation is consistent with the results of others (Biely et al., 1974; Boivin et al., 1998). The increase of material released into the culture might be correlated to the increase in the mannoprotein content of the cell walls. Numerous authors have shown that the cell walls become richer in mannoproteins during growth and maturation (Cassone et al., 1978; Valentin et al., 1987; De Nobel et al., 1990). As a consequence of these changes in the cell wall architecture, more mannoprotein material and, hence, more polymeric mannose might be released into the culture supernatant during the transition and stationary phases.

In all growth phases except decline phase, the release of polymeric glucose appeared to parallel that of polymeric mannose. During the decline phase when the number of viable cells decreased, the release of polymeric glucose ceased whereas the release of polymeric mannose continued. These results suggest that the release of most of the polymeric glucose was due to active secretion by the living cells. Whether the release of polymeric mannose was active or passive during the different phases of growth cannot be elucidated from these data. The data do indicate, however, that the increase of polymeric mannose in the culture supernatant observed during decline phase might be a passive event associated with cell death. At this early stage of cell death, the process of autolysis and enzymatic cell wall degradation had presumably not started (Charpentier and Feuillat, 1993) and thus is unlikely to explain this increasing release of polymeric mannose. However, the dying or dead cells might undergo a passive release or leakage of polymeric mannose into the culture as this material was no longer needed for the cell wall development.

Recent work showed that haze protective material (HPM) was only loosely associated with the cell wall (Dupin, 1997, Dupin et al. 2000). Thus this material could be easily released into the culture. The presence of other high M_r mannoproteins in the culture fluid, such as α-agglutinins (Sijmons et al., 1987), has been reported even though these particular mannoproteins are classified as cell wall mannoproteins. The ease of release of extracellular HPM is also consistent with the observation that HPM, like the α-agglutinins, is located on the wall surface (Dupin et al., 2000).

HPM was not detected in the first half of the exponential cell growth in the culture supernatants of all fermentations conducted here but was detected during the late exponential phase. The release of extracellular HPM into the culture during cell growth might be associated with cell wall synthesis of budding cells. Lipke and Ovalle (1998) suggest that most yeast mannoproteins and the enzymes involved in cell wall synthesis are secreted at the bud site. It is possible that, as more mannoproteins were integrated in the cell wall, more of them were released into the culture as well. As the amount of HPM released into the culture increased during the transition period between the exponential and stationary phases, the release of extracellular HPM could also be related to cell wall maturation.

HPM was also released from dead cells after 8 weeks storage of the culture supernatant on yeast lees (Table

3). Following cell death, the process of autolysis is responsible for a gradual enzymatic cell wall degradation (Charpentier and Feuillat, 1993). After 8 weeks on yeast lees at 18 °C, the process of autolysis may have begun and could explain the release of HPM observed at this stage.

The "specific activity" of HPM secreted by yeast was similar to that shown by HPM extracted from yeast in previous work (Dupin et al., 2000): at addition rates of 500 mg/L, HPM from both sources showed above average haze protective activity. Yeast invertase, the mannoprotein studied as a model haze protective mannoprotein, had slightly greater "specific activity". In this study and one reported by Moine-Ledoux and Dubourdieu (1999), an addition of 300 or 250 mg/L, respectively, to wine showed above average haze protective activity. At these addition rates, a fragment of invertase produced by proteolysis in the laboratory from purified invertase had even greater activity whereas a commercial preparation of this fragment from yeast cell walls showed only average activity (Moine-Ledoux and Dubourdieu, 1999).

The precise mechanism of action of HPM is not known. With a crude HPM fraction isolated from wine it was observed that a decrease in wine turbidity due to an increasing concentration of added HPM was accompanied by a decrease in haze particle size (Waters et al., 1993). The relationship between turbidity and concentration of HPM was exponential rather than linear (Waters et al., 1993). Haze protection was investigated further in this study using yeast invertase, a mannoprotein with haze protective activity, as a model. The nonlinear relationship between haze reduction and concentration of invertase was similar to that observed previously with a crude HPM fraction (Waters et al., 1993) and with various other macromolecules with haze protective activity (Pellerin et al., 1994).

While invertase was able to reduce the visible turbidity resulting from protein precipitation, analysis of the haze and wine after haze induction showed that invertase did not change the amount or type of protein precipitating. All grape derived protein precipitated, and no grape-derived proteins were present in the wine after heating regardless of the addition rate of invertase. This phenomenon was also seen with a crude HPM fraction isolated from wine (Waters et al., 1993). Invertase itself was not present in the haze but remained soluble in the wine. These data suggest that invertase, and presumably other HPM, decrease haze formation by competing with grape derived proteins for some unknown factor(s) in wine required to form large highly light scattering protein aggregates that are responsible for haze.

CONCLUSIONS

Material showing above average haze protective activity was isolated from a culture of *Saccharomyces cerevisiae* Maurivin PDM cells by ultrafiltration or ethanol precipitation. Neither the ultrafiltered nor the precipitated material needed further purification to show satisfactory activity. This finding was in contrast to results of experiments on whole cells or cell walls described by Dupin et al. (2000), where it was necessary to enrich the extracts in mannoproteins to detect HPM. Ethanol precipitation of the extracellular material resulted in a lower yield than the use of ultrafiltration, but the precipitated fraction showed higher haze protective activity. Thus, although ethanol precipitation is a time-consuming process, it is an efficient method to recover HPM. The collection by ethanol precipitation of extracellular material released by cells in the late growth phases could, therefore, be the basis of a method for scaled-up production of HPM. The use of cells grown on mannose instead of glucose also resulted in an increased yield of HPM. The best yields of mannoproteins from the fermentations conducted in this work were still relatively low (less than 20 g/kg of wet cells), and further work is need to develop practical techniques for the large scale production of HPM.

The mechanism of haze protection appeared to be one of competition between wine proteins and HPM for other wine component(s). We propose that these unknown wine component(s) are required for the formation of large aggregates of denatured protein that scatter light and make the wine appear hazy. An understanding of these unknown components is vital to the appropriate use of HPM and may allow us to devise oenological practices which eliminate these other compounds rather than using bentonite to remove the proteins.

ABBREVIATIONS USED

AGP, arabinogalactan protein; BSA, bovine serum albumin; HPM, haze protective mannoprotein material; M_r, relative molecular mass; CDGJM, chemically defined grape juice medium; PDM, Prise de mousse; PMI, phosphomannose isomerase.

ACKNOWLEDGMENT

We thank Holger Gockowiak and Ken Pocock of the Australian Wine Research Institute for help and guidance with the microbiology aspect of this work and for protein analysis, respectively. Wine samples were donated by Orlando Wyndham Wines (Rowland Flat, SA, Australia).

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Received for review February 25, 2000. Accepted May 16, 2000. I.V.S.D. was supported by an Overseas Postgraduate Research Scholarship from the University of Adelaide. The Grape and Wine Research and Development Corp. provided additional financial support.

JF0002443