Saccharomyces cerevisiae Mannoproteins That Protect Wine from Protein Haze: Evaluation of Extraction Methods and Immunolocalization

Isabelle V. S. Dupin,[†,§,‡] Vanessa J. Stockdale,[†,§] Patrick J. Williams,[†] Graham P. Jones,[§] Andrew J. Markides,[§] and Elizabeth J. Waters*[,†]

The Australian Wine Research Institute, PO Box 197, Glen Osmond, South Australia 5064, Australia, and Department of Horticulture, Viticulture and Oenology, University of Adelaide, Glen Osmond, South Australia 5064, Australia

Yeast-derived haze-protective mannoprotein material (HPM) offers protection to white wines from commercially unacceptable turbidities. HPM extraction methods have been evaluated using three winemaking strains of *Saccharomyces cerevisiae*. Digestion with Zymolyase of cells pretreated with DTE and EDTA gave the greatest yields of active material. Heat treatment of cells with SDS also released active material but the quantities were low. Treatment of the cells in an autoclave or with a French pressure device was less effective. A detailed study was conducted on the strain Maurivin PDM. SDS was not necessary to extract HPM from PDM; boiling the cells for 5 min in Tris buffer was sufficient. HPM could also be extracted with EDTA during the pretreatment of the cells prior to Zymolyase digestion. The data suggest that HPM was noncovalently linked to other cell wall components and loosely associated with the cell wall. An immunological investigation showed that a specific mannoprotein with haze-protective activity, HPF1, was located primarily on the outermost and innermost layers of the cell wall.

Keywords: *Yeast cell wall, mannoprotein extraction, haze-protective material, SDS, Zymolyase, EDTA, DTE, Saccharomyces cerevisiae, electron microscopy, immuno-gold labeling*

INTRODUCTION

Wines can be visually marred by hazes and sediments. One of the major causes of haziness in white wines is the precipitation of naturally occurring "heat unstable" grape proteins (Paetzold et al., 1990; Waters et al., 1991). To minimize the formation of this haze, winemakers usually lower the concentration of protein through the use of bentonite, a montmorillonite clay. This procedure is said to lower wine quality because it removes aroma components (Miller et al., 1985; Puigdeu et al., 1996). In addition, a significant loss in wine volume occurs as a result of the bentonite lees. Other methods such as ultrafiltration (Voilley et al., 1990) or the use of peptidases to degrade the heat unstable grape proteins (Waters et al., 1992, 1995) are not yet commercially viable.

We have isolated a high M_r mannoprotein called haze-protective factor (HPF1) from wine (Waters et al., 1993, 1994) that is able to prevent visible wine protein haze formation. This mannoprotein showed haze-protective activity against wine proteins and BSA when either was heated in white wine (Waters et al., 1993). Amino acid sequence analysis has since identified a putative structural gene in the *Saccharomyces cerevisiae* genome for HPF1 (Waters, unpublished work). Another high M_r yeast mannoprotein with haze-protective activity (HPF2) has since been isolated from a fermentation of chemically defined grape juice medium by a winemaking strain of *S. cerevisiae*. A putative structural gene for HPF2 has also been identified in the *S. cerevisiae* genome (Stockdale, Waters, Williams, and Fincher, unpublished work).

Independent confirmation of the haze-protective effects of yeast mannoproteins was provided by Ledoux et al. (1992) and Moine-Ledoux and Dubourdieu (1998, 1999). This work showed that wine aged on yeast lees had lower haze potential and bentonite requirements for stability than wine aged without lees but containing the same level of protein. In addition, a mannoprotein fraction isolated from yeast cell walls by enzymatic treatment was shown to protect white wine against protein haze (Ledoux et al., 1992). In contrast to the work described above, the active component from the enzymatically released fraction was of low M_r and identified as a 32 kDa fragment of yeast invertase (Moine-Ledoux and Dubourdieu, 1999). A process to obtain the active component by enzymatic digestion of yeast cell walls with a commercial β-glucanase preparation has been described (Moine-Ledoux and Dubourdieu, 1999).

Other glycoproteins have also been shown to exhibit haze-protective activity. These include yeast invertase (McKinnon, 1996), a wine arabinogalactan-protein (Waters et al., 1994b), and an apple arabinogalactan-protein (Pellerin et al., 1994).

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

* To whom correspondence should be addressed. Fax: (+618) 8303 6601. E-mail: ewaters@waite.adelaide.edu.au.
† The Australian Wine Research Institute.
‡ Present address: Staatliche Lehr- und Forschungsanstalt, Neustadt a.d. Weinstrasse, Germany.
§ University of Adelaide.

10.1021/jf9905020 CCC: $19.00
Published on Web 03/24/2000

precipitating, but rather decreased the particle size of the haze (Waters et al., 1993). An unpurified yeast mannoprotein fraction, at the highest level examined, decreased wine haze particle size to 5 μm and the haze was barely detectable with the naked eye (Waters et al., 1993).

Clearly, haze-protective mannoprotein material (HPM) offers the wine industry a potential alternative to bentonite fining. The most effective procedures for the extraction and recovery of HPM require investigation and the location of HPM and its relation to the yeast cell envelope needs to be confirmed. This information will assist in the development of HPM as a commercially viable wine processing aid.

In this paper, we describe physical, chemical, and enzymatic methods for HPM from *Saccharomyces cerevisiae*. Data obtained from this study allowed us to devise a model describing how HPM is associated with the yeast cell wall. In addition, immunological techniques have been used to localize a specific mannoprotein with haze-protective activity, HPF1, in the cell wall.

MATERIALS AND METHODS

Strains, Media and Growth Conditions. *Saccharomyces cerevisiae* Maurivin PDM (Champagne origin) was obtained from Mauri Foods yeast group (Sydney, Australia) while *Saccharomyces cerevisiae* AWRI 65 (a flocculent yeast) and AWRI 85 (a French Champagne wine yeast) were sourced from the Australian Wine Research Institute culture collection (Adelaide, Australia). Chemically defined grape juice medium (CDGJM), adapted from that described by Henschke and Jiranek (1993) contained glucose (200 g/L), potassium hydrogen tartrate (2.5 g/L), L-malic acid (3 g/L), $MgSO_4 \cdot 7H_2O$ (1.23 g/L), K_2HPO_4 (1.14 g/L), $CaCl_2$ (0.33 g/L), citric acid (0.2 g/L), myo-inositol (100 mg/L), pyridoxine HCl (0.78 mg/L), nicotinic acid (3.125 mg/L), calcium pantothenate (1.95 mg/L), thiamin HCl (1.055 mg/L), riboflavin (78 μg/L), biotin (24 μg/L), NH_4Cl (1.76 g/L), $MnCl_2 \cdot 4H_2O$ (198.2 μg/L), $ZnCl_2$ (135.5 μg/L), $FeCl_2$ (31.96 μg/L), $CuCl_2$ (13.6 μg/L), H_3BO_3 (5.7 μg/L), $Co(NO_3)_2 \cdot 6H_2O$ (29.1 μg/L), $NaMoO_4 \cdot 2H_2O$ (24.2 μg/L), $KClO_3$ (10.8 μg/L) and was adjusted to pH 3.5.

Yeast strains were maintained on yeast peptone dextrose slopes (DIFCO Laboratories, Detroit, MI). Using maintenance cultures, individual yeast strains were inoculated into 10 mL of CDGJM and subsequently into larger volumes of CDGJM using an inoculum of exponentially growing cells at a rate of 5% (v/v) at 25 °C under agitation. Upon reaching exponential phase, the final propagated culture (500 mL) was transferred to 9.5 L of CDGJM.

Yeast growth was monitored by measuring the absorbance of the culture at 650 nm (A_{650}). Cultures were grown at 25 °C to late exponential phase (7.5−11.5 g/L, wet cell weight) or to stationary phase (16 g/L, wet cell weight). The cell morphology, including budding, was assessed by phase contrast microscopy (×1000). The cells were recovered by centrifugation (18 000 *g*, 10 min, 5 °C), washed with one volume of water (5 times) and either used immediately or stored at −20 °C.

Mechanical Disruption with a French Pressure Cell. Cells (14 g wet cell weight) were suspended in chilled Tris HCl buffer (70 mL, 50 mM, pH 7.5) containing phenylmethylsulfonyl fluoride (1 mM). Up to four passages of the yeast suspension at 4 °C and at constant speed (3 mL/min) through the press (cell pressure of 140 MPa) were necessary to obtain 95% of cell disruption (as observed by phase contrast microscopy). The cell debris was recovered by centrifugation (48 000*g*, 15 min, 5 °C), washed with water (50 mL, twice) and stored at −20 °C. The supernatants were collected and filtered through a 0.45 μm membrane.

Pretreatment and Zymolyase Digestion of the Cell Wall ("Full Zymolyase Treatment"). Cells (4.5 g wet cell weight) were initially pretreated in Tris HCl buffer (15 mL, 100 mM, pH 8) containing DTE (5 mM) and EDTA (5 mM) at 28 °C for 30 min in a shaking water bath (300 rpm, model OWD 1412, Paton Scientific, Adelaide, Australia). The cell pellet was recovered by centrifugation (48000*g*, 10 min, 5 °C) and washed with water (15 mL, twice). The supernatants from the pretreatment and the washings were pooled and dialyzed against distilled water (6 L, changed six times) at 4 °C. The cells were resuspended in the same Tris HCl buffer as above (100 mM, pH 8, 5 mM DTE and 5 mM EDTA) containing Zymolyase [2% (w/v), Zymolyase 100T, ICN Pharmaceuticals Inc., Sydney, Australia, 6700 lytic units/g wet cells (one unit will produce a ΔA_{800} of 0.001/min at pH 7.5 and 25 °C using a suspension of brewers yeast in a reaction volume of 3 mL)] and incubated at 28 °C for 60 min in a shaking water bath as described above. After incubation, the suspension was centrifuged and washed as described above. The supernatants from the Zymolyase digestion were collected and pooled with those from the pretreatment and then filtered through a 0.45 μm membrane. The insoluble material was discarded.

Subtreatments Related to the Full Zymolyase Treatment of the Cells. Cells were initially pretreated, as described in the previous section, with DTE and EDTA. The supernatants from the pretreatment and the washings were pooled and filtered through a 0.45 μm membrane but not dialyzed. The cells were treated with Zymolyase as described above. The supernatants from the Zymolyase digestion were filtered through a 0.45 μm membrane but not pooled with the supernatant from the pretreatment and washings.

Extraction with Hot SDS. Cells, or cell debris after mechanical disruption (14 g wet weight), were suspended in Tris HCl buffer (70 mL, 10 mM, pH 7) containing SDS [2% (w/v)] and boiled for 5 min with manual shaking. The suspension was centrifuged (48000*g*, 10 min, 5 °C) and the pellet was washed with water (70 mL, twice). The supernatants were dialyzed against distilled water (20 L, changed three times) at 4 °C and ultrafiltered (YM 10 membrane, 10 kDa molecular weight cutoff, Amicon, Danvers, MA). The retentate was kept.

Autoclave Treatment. Cells (15 g wet cell weight) were suspended in sodium citrate buffer (100 mL, 20 mM, pH 7) and autoclaved at 105 °C for 60 min (modified from the method of Peat et al., 1961). The cell debris was recovered by centrifugation (48000*g*, 10 min, 5 °C) and washed with 100 mL of water (twice). The supernatants and the washings were pooled and filtered through a 0.45 μm membrane. The cell debris was discarded.

Concanavalin-A (Con-A) Affinity Chromatography. All solutions were degassed before use. Filtered supernatants obtained from the extractions were diluted 10-fold in starting buffer [Tris HCl buffer (20 mM, pH 7.4) containing NaCl (0.5 M), $CaCl_2$ (0.5 mM), $MgCl_2$ (0.5 mM), and $MnCl_2$ (0.5 mM)], and loaded at 1 mL/min onto a Con-A column (HR 16/50 column, Pharmacia, Sydney, Australia) equilibrated with starting buffer. Unbound material was eluted with starting buffer (approximately 10 column volumes) at 1 mL/min. The material retained by the Con A column was eluted with elution buffer [starting buffer containing methyl-α-D-mannoside (0.1 M)] at 1 mL/min. Protein was detected by monitoring the absorbance at 280 nm on a Waters 440 absorbance detector (Waters Millipore, Milford, MA).

The fraction containing the material eluted by methyl-α-D-mannoside was desalted by ultrafiltration in a 400 mL capacity stirring cell, equipped with a YM 10 membrane, at 4 °C under a nitrogen pressure of approximately 400 kPa. The retentate was collected, freeze-dried, and weighed.

Micromethod for the Measurement of the Heat-Induced Haze in Wine (Heat Test). The effects of mannoprotein additions on the protein haze potential of wines were determined by a modification of the micromethod described by Waters et al. (1991). Wine was commercially produced from *Vitis vinifera* L. Muscat of Alexandria grapes, ultrafiltered (Amicon YM 10 membrane) to remove grape proteins and supplemented with bovine serum albumin (BSA) to give a final protein concentration of 125 mg/L. Aqueous solutions of mannoproteins (0−15 μL, made up to 15 μL with water, final extract concentration of 0, 500, 1000, or 2000 mg/L on dry

weight basis) were added to the wine (180 μL). After being mixed and sealed, the samples were heated for 1 h at 80 °C and left on ice for 1 h. After 20 min at room temperature, 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., Sunnyvale, CA). Values were corrected by subtraction of the absorbance at 490 nm for a control (no BSA or mannoprotein added before heat testing).

Production of Polyclonal Antibodies. Antibodies against purified HPF1 (Waters et al., 1994) were obtained from a New Zealand White rabbit. The purity of the sample after storage at −20 °C and before immunization was assessed by gel permeation chromatography as described by Waters et al. (1994). For immunization, HPF1 (80 μg) in sterile saline solution (1 mL) was combined with phosphate-buffered saline (PBS, 0.5 mL) and mixed with complete Freund's adjuvant (1.5 mL). The water in oil emulsion was injected intramuscularly at two separate sites. After 3 weeks, the same protocol was applied using incomplete Freund's adjuvant (dilution 1:1). The emulsion was administered subcutaneously at six separate sites. Four weeks after the last injection, 1 mL of the immunogen solution without adjuvant was injected intravenously. One week later, the rabbit was test bled and the serum separated to check the specificity of the antibodies produced and to detect any cross-reactivity. As the test procedure indicated no cross-reactivity, the rabbit was sacrificed and the collected serum was retested for specificity and cross-reactivity (see below). The serum was stored in aliquots (10 mL) at −20 °C before use. An immunoglobulin G (IgG) fraction was prepared from the serum by protein A affinity chromatography according to the procedure of Ey et al. (1978).

Test of Immunospecificity and Cross-Reactivity by Ouchterlony's Immunodiffusion Assay. A gel double diffusion assay was performed according to Ouchterlony (1949). HPF1 and potential cross-reacting antigens (mannans, invertase, BSA, all from Sigma Chemical Co., St. Louis, MO) were placed on wells cut into a horizontal 1% (w/v) agarose gel (Type II: medium EEO, Sigma). The test at the stage of prebleeding was performed with antiserum at 1:5 dilution. HPF1 and the potential cross-reacting antigens tested were used at a concentration of 1 mg/mL. Diffusion of antibodies and antigens occurred overnight at room temperature. The test carried out after the final bleeding was performed as above except that the dilution of the antiserum used was 1:2.

Test of Immunospecificity and Cross-Reactivity by Electrophoresis in Agarose Gels and Immunoblotting. Gels containing 1.8% or 1.4% of agarose were used in this study and run as horizontal submerged slabs. Agarose [1.4% or 1.8% (w/v), Type II: medium EEO, Sigma] was added to gel buffer [20 mL, pH 8.6, 970 mM Tris, 280 mM glycine, 58 mM calcium lactate, 0.01% (w/v) SDS] and dissolved by heating. The solution was then poured into the gel casting, and an eight-well comb was placed in the top of the gel. The gels were allowed to set for 30 min. The gel dimensions were 95 × 75 × 2 mm. Samples (10 μg) were diluted in sample buffer and then loaded into the wells. Sample buffer was prepared by combining water (400 μL), gel buffer (150 μL), glycerol (100 μL), and bromophenol blue (50 μL, 0.2% (w/v). Gels were run in running buffer [50 mM Tris, 380 mM glycine, 10% (w/v) SDS] at a constant current of 70 mA until the bromophenol tracker dye was 5 mm from the bottom of the gel (about 4 h). Transfer of that material which migrated on the agarose gel to a nitrocellulose membrane (pore size: 0.45 μm, Schleicher and Schuell, Dassel, Germany) was done using BioRad Mini Trans Blot Electrophoretic Transfer Cell following the manufacturer's instructions (BioRad Laboratories, Sydney, Australia). After completion of the transfer to nitrocellulose, the membrane was immunologically tested using the Bio Rad Immuno Blot Assay Kit (BioRad Laboratories) according to the manufacturer's instructions. The rabbit antiserum was diluted to 1:500 or 1:1000.

Direct Agglutination Assay. Fresh Maurivin PDM yeast cells grown in CDGJM to late exponential phase were harvested and washed (three times) in PBS. Cells [0.5% (v/v)] were resuspended in PBS.

The agglutination assay was carried out in a flat bottom microtitration plate. The serum (100 μL) was added to the top row and serially diluted in PBS (100 μL) by half along the row (final well volume of 100 μL, 12 wells in total). The preimmune serum was similarly serially diluted by half along the wells of the second row and was referred to as the preimmune serum control. The third row contained only PBS (100 μL) and was referred to as the serum-free control row. The yeast suspension (50 μL) was added to all rows. Each row was done in duplicate and contained a final volume of 150 μL. The microtitration plate was briefly mixed and left 2 h at room temperature. The formation of macroscopic clumps was assessed with the naked eye over a white background.

Immunoelectron Microscopy. Fresh Maurivin PDM yeast cells grown in CDGJM to late exponential phase were harvested, washed with water (three times), and fixed in 0.25% (v/v) glutaraldehyde in PBS containing sucrose [4% (w/v)] for 12 h. Cells were washed in PBS containing sucrose (twice, 30 min each time) and then dehydrated by successive washings in 70% (v/v) ethanol (twice, 30 min each time), 90% (v/v) ethanol (twice, 30 min each time), 95% (v/v) ethanol (twice, 30 min each time), and 100% ethanol (v/v) (twice, 30 min each time and once for 60 min). Dehydrated cells were preembedded in a mixture of 50% (w/v) LR White Resin (Probing & Structure, Brisbane, Australia)/50% (v/v) absolute ethanol for 15 h at 4 °C and in 100% LR White Resin (three times, 2 h each time) at room temperature. After the third change of resin, cells were embedded in 100% LR White Resin and placed in an oven at 50 °C for 24 h to allow the resin to polymerize. Ultrathin resin sections (thickness around 50 nm) were cut with a Reichert Ultracut E (Reichert, Germany) at room temperature and collected on collodion coated nickel grids (3 mm diameter).

For the immunogold labeling of the ultrathin sections, preliminary assays were carried out to determine the appropriate dilution range of primary antibody or gold probe in order to get minimum background on all sections and no labeling of the negative control sections (see below). During the procedure, the grids were treated by floating them on top of drops (15−20 μL) of reagent dispensed onto sheets of Parafilm. The grids were treated with glycine (0.02 M) in PBS for 20 min, blotted onto filter paper, and then floated on antibody buffer (PBS with ovalbumin [1% (w/v)], Tween-20 [0.5% (v/v)] and Triton-X-100 [0.1% (v/v)]) for 20 min. After blotting onto filter paper, the grids were placed on the primary antibody solution (IgG fraction diluted to 1:400 with antibody buffer) for 15 h at 4 °C. The negative controls were prepared as follows. To test for nonspecific binding by the primary antibodies (IgG fraction), the grids were placed onto a solution containing the preimmune serum (diluted to 1:400 with antibody buffer) instead of IgG fraction for 15 h at 4 °C. To test for nonspecific binding by the gold probe, the grids were floated on antibody buffer in place of the primary antibody solution for 15 h at 4 °C. All sections were rinsed with PBS containing ovalbumin [1% (w/v), six times, 5 min each time] and blotted onto filter paper. The grids were then incubated with a solution of Autoprobe EM protein A G10 [colloidal gold: 10 nm mean diameter, Amersham International, Great Britain, 2% (v/v)] for 60 min and rinsed with PBS containing ovalbumin (six times, 5 min each time) and water (four times).

For the staining procedure, sections were placed on uranyl acetate [5% (w/v) stabilized with glacial acetic acid and centrifuged before use] for 10 min and washed with water (four times). The sections were then floated on lead citrate reagent [1.3 g lead nitrate ($Pb(NO_3)_2$), 1.8 g sodium citrate, 8 mL 1 N NaOH in 50 mL water, centrifuged before use] for 5 min and washed with water (four times).

The stained sections were examined using a Philips CM 100 transmission electron microscope.

Table 1. Yield and Haze-Protective Ability of Mannoprotein Material Obtained with Different Methods of Extraction Applied to Cells of *Saccharomyces cerevisiae* Strains Maurivin PDM, AWRI 65, and AWRI 85 in Late Exponential Phase after Freeze−Thawing

	mannoprotein yield (% w/w)[a]			haze-protective ability (% haze)[b]		
treatment	Maurivin PDM	AWRI 65	AWRI 85	Maurivin PDM	AWRI 65	AWRI 85
(1) full Zymolyase treatment	1.28	0.80	1.65	$34^{mn\,c}$	69	36^{m}
(2) French press	0.52	0.83	0.40	72^{o}	117	94^{n}
(3) autoclave	0.57	0.51	0.61	57^{no}	82	70^{o}
(4) SDS treatment	0.20	0.10	0.11	26^{m}	85	32^{m}
F^d				*	ns	**

[a] Results are expressed as % (w/w) of mannoprotein material extracted (dry weight) per wet weight of cells used for the extraction. Values are the means of at least two independent experiments. [b] Haze as a percentage of the initial haze value (as observed with no mannoprotein extract added) seen at a concentration of 0.5 mg of mannoprotein material per mL of wine in the micromethod for the measurement of the heat-induced haze. Values are the means of at least two independent experiments. [c] Means in the same column with different superscript letters where statistically significantly different at the 5% level according to the Student *t* test. [d] Significance of the *F* value: * = $p < 0.05$; ** = $p < 0.01$; ns = not significant.

RESULTS AND DISCUSSION

Evaluation of Methods for Extracting Haze-Protective Material. *Extracted Mannoprotein Yields*. Four different methods of extraction of HPM from *Saccharomyces cerevisiae* cells were evaluated. Mannoproteins contained in the crude extracts from these cells were isolated by affinity chromatography on the lectin Con-A (this lectin has high affinity for mannoproteins; So and Goldstein, 1968), and the resulting mannoprotein fractions were desalted and lyophilized. The dry weights of the mannoprotein fractions represent the weight of the mannoproteins in the original extract and thus the yield.

In general for the three yeasts tested, the full Zymolyase treatment was the most effective treatment for releasing mannoproteins (Table 1). Zymolyase has both β-glucanase and protease activity and is thought to attack the glucan network of the cell wall, thus releasing mannoproteins interspersed within or linked to this network (Pastor et al., 1984; Elorza et al., 1985; Molloy et al., 1989). The action of Zymolyase on the cell wall would also release periplasmic and, because the extraction was performed without an osmotic stabilizer, cytoplasmic material. Cytoplasmic mannan, however, only accounts for 0.5−1.5% of the total cellular mannan (Katohda et al., 1976) and thus its contribution to the total mannoprotein yield is expected to be small. Since Zymolyase releases material from the cell wall matrix and from the periplasmic space, and both these sites have a high proportion of mannoproteins, the mannoprotein yield from this method was expected to be relatively high. This was observed (Table 1).

Intermediate yields of mannoproteins were obtained after mechanical disruption of the cells with a French press or after autoclaving the cells (Table 1). Both these methods are reported to release material from the cytoplasm and periplasmic space as well as from the cell wall (Arnold, 1972; Fleet, 1991). These methods are probably less effective than Zymolyase in releasing material from these sites because they primarily disrupt physical barriers and not covalent linkages.

In contrast to the other methods, treatment with SDS resulted in low yields of mannoproteins (Table 1). SDS is reported to have a limited effect on whole cells (Horvath and Riezman, 1994) and only the surface directly in contact with SDS would be extracted. Therefore, the small amount of material extracted is probably originating from the outer cell wall alone. When the inner surface of the cell wall of PDM cells was exposed by subjecting the cells to the French press before extraction with SDS, 6 times more material was released (data not shown).

Apart from the mechanical disruption procedure, higher mannoprotein yields were obtained from PDM and AWRI 85 cells than from AWRI 65 cells, particularly for the full Zymolyase extraction (Table 1). Flocculent yeasts such as AWRI 65 have a cell wall richer in mannoproteins and glucans compared to yeast with nonflocculating properties (Al-Mahmood et al., 1987, Saulnier et al., 1991) and there are significant differences between these groups of yeasts in the structure and molecular weight of the cell wall mannoproteins (Amri et al., 1982; Bellal et al., 1995). It is possible that these compositional changes produce structural differences in flocculent yeast compared to nonflocculent yeast that impair the activity of Zymolyase and result in lower extraction yields of mannoproteins from flocculent yeast. This hypothesis would need to be confirmed by examining a greater number of yeast strains.

Haze-Protective Ability of the Mannoprotein Material Extracted. The haze-protective activity of the different extracts was tested by comparing the level of haze produced from heating the protein, BSA, in wine with and without the mannoprotein extracts. Previous studies have shown that BSA reacts similarly to wine proteins under these conditions (Waters et al., 1993). Because of the variability of the heat test (up to 10% standard deviation), the activity of the extracts has been classified into four broad categories. First, at mannoprotein concentrations of 0.5 g/L, extracts classified as having "above average", and "average", activity reduced the initial 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 extracts increased beyond 0.5 g/L, the percentage of haze either decreased further or remained constant. Extracts classified as having "borderline" activity reduced the haze to between 60% and 90% of the initial haze value at a mannoprotein concentration of 0.5 g/L but the percentage of haze tended to increase with higher extract concentrations. Extracts classified as having no activity gave haze values that were greater than 90% of the initial haze value. The protective activity of the extracts and a statistical evaluation of the results are given in Table 1.

For PDM and AWRI 85, both the full Zymolyase and SDS treatments released material with above average haze-protective activity whereas the autoclave treatment of the cells of these two strains released material with only average or borderline activity (Table 1).

Borderline or no activity was present in the French pressed extracts from these two strains, apart from the PDM cells which were treated with SDS after disruption using a French pressure cell. In this latter case, average activity was observed (data not shown).

Moine-Ledoux and Dubourdieu (1999) prepared similar extracts to those obtained here by treating a cell wall preparation of a winemaking strain of *S. cerevisiae* with a commercial enzyme preparation containing β-glucanases and proteases (Glucanex, similar to Zymolyase treatment used here) or extracting the cell wall preparation with heat using the method of Peat et al. (1961) (similar to the autoclave method used here). Only broad comparisons between the haze-protective activities of the extracts prepared by Moine-Ledoux and Dubourdieu (1999) and of those described here can be made because the heat test conditions, methods to quantify haze, and the unstable protein type and concentration in the wine were different. Nevertheless, both studies gave similar results: the Glucanex extract of Moine-Ledoux and Dubourdieu (1999) showed an average level of haze-protective activity at an addition rate of 0.25 g/L and the extract prepared from either PDM or AWRI 85 by Zymolyase treatment here gave above average activity at twice this addition rate. Similarly, at the same addition rate of 1 g/L, both the heat extract of Moine-Ledoux and Dubourdieu (1999) and the autoclave extract prepared here from either PDM or AWRI showed an average level of haze-protective activity (data not shown).

In contrast to the other yeast strains examined here, no activity of consequence was observed for any extract from AWRI 65 (Table 1). It is possible that, concurrent with other changes in mannoprotein composition of these yeast as described above, the concentration of HPM in extracts from flocculent yeast is also different to that in extracts from nonflocculent yeast. Alternatively, HPM may be more difficult to extract from flocculating yeast compared to nonflocculating yeast due to the previously described changes in the cell walls. As described above, this hypothesis would need to be confirmed by examining a greater number of yeast strains.

Reagents or Conditions Responsible for the Release of HPM during the SDS Treatment and the Full Zymolyase Treatment on Maurivin PDM. Among the methods tested and described above, the full Zymolyase and the hot SDS treatments were the most effective at extracting mannoproteins with average or above average haze-protective activity. For the full Zymolyase treatment, the mannoproteins could have been released either during the pretreatment of the cells by EDTA and DTE or during the final digestion of the pretreated cells with Zymolyase. Similarly, for the hot SDS treatment the release of haze-protective mannoproteins could be either due to the action of SDS alone, the boiling procedure, or their combined effects. Thus, the two sets of treatments were further examined to determine which components in each treatment were responsible for the release of HPM.

Zymolyase Treatment. The full Zymolyase treatment was split into two subtreatments. Cells were pretreated with DTE and EDTA, and the mannoprotein material collected was referred to as the pretreatment extract. The pretreated cells were then digested with Zymolyase and the second crop of mannoprotein material collected thus contained only material extracted by the enzyme (referred to as the Zymolyase extract).

Table 2. Yield and Haze-Protective Ability of the Mannoprotein Extracts Obtained by Various Treatments from *Saccharomyces cerevisiae* Maurivin PDM Cells in Late Exponential Phase after Freeze−Thawing

treatment	mannoprotein yield (% w/w)[a]	haze-protective ability (% haze)[b]
(1) pretreatment: combined DTE and EDTA treatments	0.87[m c]	45[m]
(2) Zymolyase treatment: 2% (w/v) Zymolyase on pretreated cells, 28 °C, 60 min	1.10[m]	81[n]
(3) DTE treatment: 5 mM DTE, Tris HCl buffer, 28 °C, 30 min	0.54[n]	171[o]
(4) EDTA treatment: 5 mM EDTA, Tris HCl buffer, 28 °C, 30 min	0.44[n]	46[m]
F[d]	*	****

[a] Results are expressed as % (w/w) of mannoprotein material extracted (dry weight) per wet weight of cells used for the extraction. Values are the means of at least two independent experiments. [b] Haze as a percentage of the initial haze value (as observed with no mannoprotein extract added) seen at a concentration of 0.5 mg of mannoprotein material per mL of wine in the micromethod for the measurement of the heat-induced haze. Values are the means of at least two independent experiments. [c] Means in the same column with different superscript letters where statistically significantly different at the 5% level according to the Student t test. [d] Significance of the F value: * = $p < 0.05$; **** = $p < 0.0001$.

The two subtreatments extracted mannoproteins to similar extents (treatments 1 and 2, Table 2). The sum of the mannoprotein material extracted by these two separate treatments was greater than that extracted by the full treatment (Table 1). Both dialysis and ultrafiltration were used to prepare the samples for the full Zymolyase treatment whereas only ultrafiltration was used to obtain the extracts from the two subtreatments. This change in procedure might explain the differences in yield observed as some material could have been lost during dialysis. Average haze-protective activity was exhibited by the pretreatment extract (Table 2). In contrast, only borderline activity was observed when the Zymolyase extract was tested. Thus, the pretreatment with EDTA and DTE specifically extracted HPM whereas Zymolyase treatment of the pretreated cells released material with no obvious haze-protective ability.

Individual extractions with EDTA and DTE were then carried out to test whether both or only one of these compounds contained in the pretreatment was responsible for the release of HPM. Both mannoprotein extracts obtained by EDTA and DTE treatments (referred to as the EDTA or DTE extracts, respectively) contained less mannoproteins than the extract from the combined pretreatment and acted differently in reducing protein haze in wine (Table 2). The DTE extract dramatically increased the level of haze. This result suggested that material with a strong haze forming ability was extracted by DTE rather than HPM. Alternatively, other mannoproteins with strong haze-inducing properties may have been simultaneously extracted with HPM and masked the haze-protective effects of HPM. In contrast to the DTE extract, the EDTA extract reduced haze to the same extent as that obtained by the combined pretreatment extract (Table 2).

Accordingly, of the three agents (EDTA, DTE, and Zymolyase) used in the full Zymolyase treatment, DTE and Zymolyase did not appear to significantly release

Table 3. Yield and Haze-Protective Ability of the Mannoprotein Extracts Obtained by SDS Treatment of Freshly Harvested *Saccharomyces cerevisiae* Maurivin PDM Cells at Late Exponential (LEP) or Stationary Phase (SP)

	mannoprotein yield (%w/w)[a]			haze-protective ability (% haze)[b]		
treatment	LEP	SP	F^c	LEP	SP	F
SDS treatment: boiling in 2%(w/v) SDS, Tris HCl buffer, 5 min	0.20	0.28	ns	26	28	ns
SDS control: boiling in Tris HCl buffer, 5 min	0.26	0.26	ns	25	24	ns
F^c	ns	ns		ns	*	

[a] Results are expressed as % (w/w) of mannoprotein material extracted (dry weight) per wet weight of cells used for the extraction and are the means from two experiments [b] Haze as a percentage of the initial haze value (as observed with no mannoprotein extract added) seen at a concentration of 0.5 mg mannoprotein material per mL of wine in the micromethod for the measurement of the heat-induced haze. Values are the means of three independent experiments. [c] Significance of the F value: * = $p < 0.05$; ns = not significant.

Table 4. Yield and Haze-Protective Ability of the Mannoprotein Extracts Obtained by EDTA Treatment of Freshly Harvested *Saccharomyces cerevisiae* Maurivin PDM Cells at Late Exponential (LEP) or Stationary Phase (SP)

	mannoprotein yield (% w/w)[a]			haze-protective ability (% haze)[b]		
treatment	LEP	SP	F^c	LEP	SP	F
EDTA treatment: 5 mM EDTA, Tris HCl buffer, 28 °C, 30 min	0.05	0.03	ns	45	95	*
EDTA control: Tris HCl buffer, 28 °C, 30 min	0.04	0.03	ns	67	135	***
F	ns	ns		*	*	

[a] Results are expressed as % (w/w) of mannoprotein material extracted (dry weight) per wet weight of cells used for the extraction and are the means from two experiments [b] Haze as a percentage of the initial haze value (as observed with no mannoprotein extract added) seen at a concentration of 0.5 mg of mannoprotein material per mL of wine in the micromethod for the measurement of the heat-induced haze. Values are the means of three independent experiments. [c] Significance of the F value: * = $p < 0.05$; *** = $p < 0.001$; ns = not significant.

HPM. EDTA therefore appears to be the major agent extracting HPM.

Freeze−Thawing. Freezing was commonly used to store yeast cells prior to extraction. To investigate the possible effect of the freeze−thawing cycle on the release of HPM, the washing liquid from frozen cells was examined for both mannoproteins and haze-protective activity. Low levels of mannoproteins (0.07% w/w) were present in the washing liquid and these mannoproteins had only borderline activity (data not shown). It is postulated that the physical processes of freeze−thawing were responsible for the release of these mannoproteins. Since it was also possible that the freeze−thawing cycle could affect the extractability of the remaining cell walls of the thawed cells, further experiments with SDS and EDTA were carried out on freshly harvested cells (Tables 3 and 4, respectively) and compared with the results from frozen cells. These are described below.

SDS Treatment. The SDS treatment was applied to freshly harvested cells in late exponential phase. As observed for frozen cells in the same growth phase (Table 1), the yield of mannoproteins was low but the extract had above average activity (Table 3). The mannoprotein extract obtained in the absence of SDS (referred to as the SDS control extract: boiling whole cells in 10 mM Tris buffer, pH 7, for 5 min) also gave a similar yield and haze-protective ability to that shown by the SDS extract. These results suggested that SDS, an anionic detergent, was not specifically needed for HPM extraction.

EDTA Treatment. The amount of material extracted by EDTA treatment of fresh cells in late exponential phase compared to that simply extracted by the buffer (100 mM Tris buffer, pH 8, for 30 min at 28 °C) was negligible (Table 4). The haze-protective activity of the two extracts was, however, different. The material extracted without EDTA showed only borderline activity (Table 4). The material extracted with EDTA from these fresh cells in late exponential phase showed average activity (Table 3).

The yield of mannoproteins from the EDTA treatment of fresh cells in late exponential phase was dramatically lower than that from freeze−thawed cells in late exponential phase (Table 2), suggesting that the freeze−thawing cycle may facilitate the action of EDTA. Despite the difference in yield, the haze-protective activity of the two extracts was similar.

Effect of the Yeast Growth Phase on the Extraction of Haze-Protective Material. The SDS treatment and its control were applied to freshly harvested cells in late exponential phase (as described above) or stationary phase. The yields of mannoprotein and the activity of the extracts were similar (Table 3). These results suggested that the phase of cell growth was not important to the extraction of HPM by boiling whole cells.

The yield of material from the EDTA treatment and its control was unaffected by the growth phase of the cells and was uniformly low (Table 4). None of the extracts from the cells in stationary phase showed haze-protective activity (Table 4). Since HPM is known to be present in the cells in stationary phase (as it was extracted by the SDS treatment), the lack of any haze-protective ability of the EDTA extract from fresh stationary phase cells suggests that EDTA was not able to extract HPM from cells at this growth stage. A further possibility is that while EDTA extracted HPM from cells in stationary phase, other mannoproteins with strong haze-inducing properties were simultaneously extracted and either masked or inhibited the haze-protective effects of HPM. It is believed that the architecture of the cell wall evolves during cell maturation leading to a more structured, rigid, and less porous cell wall (De Nobel et al., 1990; Valentin et al., 1987). Because of these architectural rearrangements, the cell wall may have been more stable and resistant to the action of EDTA, thus preventing the release of HPM.

A Model for the Association of Haze-Protective Mannoprotein Material with the Cell Wall Based on the Extraction Data. The results obtained in this study suggest that HPM is not covalently linked through $\beta(1 \rightarrow 3)$ bonds to the glucan network because Zymolyase, a $\beta(1 \rightarrow 3)$ glucanase, was not needed to release HPM. HPM also does not appear to be linked to other cell wall components by disulfide bridges because DTE, a reagent able to reduce these bonds, was also not needed. Furthermore, the specific release of HPM by EDTA (a metal ion chelating agent) implies that HPM is retained in the cell wall by ionic interactions and thus only loosely associated with it. The results obtained

after examining the SDS treatment also support this hypothesis. HPM was released by simply boiling whole cells in buffer; SDS was not required. Since heat treatment destabilizes all types of noncovalent interactions including ionic and hydrogen bonds (Schwatzberg and Hartel, 1992), these data support the suggestion that HPM was only interacting with other cell components by noncovalent linkages, such as ionic bridges.

The presence of metal ions in the cell wall is reported to compensate for the negative charges of the phosphate groups present in the outer core of the structural mannoproteins, as well as those of the peptide moieties of the mannoproteins, and thus to stabilize the whole cell wall (De Nobel et al., 1989; Valentin et al., 1984). The formation of ionic bridges also contributes to cell wall cohesion. Because of its chelating properties, EDTA can extract metal ions and thus disorganize the ionic interactions within the cell wall leading to the release of cell wall components into the medium. This might explain the release of HPM during the extraction process and suggests that ionic bridges play a role in maintaining HPM within the cell wall. In addition, an extraction experiment using EDTA at pH 3 and 5.5, a pH range in which the chelating abilities of EDTA are reduced (Janson and Ryden, 1989), did not lead to the release of HPM (Dupin, 1997). This result further supports the hypothesis that the release of HPM by EDTA at pH 8 was due to the depletion of ions from the cell wall by EDTA.

Immunolocalization of HPF1 in the Cell Wall of Maurivin PDM. *Specificity and Cross Reactivity of the Polyclonal Antibodies*. Antibodies to HPF1 were raised in a rabbit using HPF1 purified by a multistep chromatographic procedure from red wine (Waters et al., 1994a). Before being used for the immunization, the purified HPF1 had been stored as an aqueous solution at −20 °C for over 12 months. The fidelity of this sample was analyzed by gel permeation chromatography and only a single peak was detected with a M_r of 420 000 as previously found by Waters et al. (1994a; data not shown). During immunization, the antiserum was tested by Ouchterlony's immunodiffusion assay to assess the specificity and cross-reactivity of the polyclonal antibodies produced (data not shown). Two continuous precipitation lines, typical for reactions of identity, appeared in the gel between the well containing the antiserum and that of the solution containing HPF1, suggesting that the solution injected into the rabbit contained two components. The two components could be two different mannoproteins of similar M_r which could not be differentiated by gel permeation chromatography. It is more likely, given the purity of the immunogen, that the two components represented two differently glycosylated forms of the same mannoprotein, a common situation with yeast mannoproteins (Trimble and Maley, 1977; Esmon et al., 1981).

There was no reaction between the antiserum and yeast invertase (containing 50% mannose), a commercial yeast cell wall mannan fraction or BSA. Additionally, after agarose gel electrophoresis and immunostaining, no color development was observed either as a smeared spot or as a band for invertase or BSA (data not shown). Because of the lack of sample, the antigenic solution containing purified HPF1 was not examined by electrophoresis, but, as a compromise, the crude extracts described above which had haze-protective activity, and presumably contained HPF1, were examined. After agarose gel electrophoresis, and immunostaining, these extracts showed two fine bands of light intensity separated by only a few millimeters in the first top quarter of the gel (very high M_r zone, data not shown). In total, these results suggest that the antiserum was specific for the purified HPF1 sample used for immunization and indicate that the purified HPF1 sample contained two high M_r mannoproteins.

Presence of HPF1 Antigenic Determinants on the Maurivin PDM Cell Surface. Interpretation of the Direct Agglutination Assay. An agglutination assay was conducted using the antiserum obtained after the immunization to examine the presence of HPF1 on Maurivin PDM yeast cells' surface. Antibodies have multiple binding valency and are able to bind at the same time to several antigenic determinants. If the determinants recognized by the antibodies are located on different cells, the antibodies create bridges between the cells. As a result, provided that a sufficient amount of antibodies is present, the cells agglutinate and form clumps visible with the naked eye.

An agglutination pattern (large circle of clumped cells in the well) was clearly visible at high concentration of the antiserum. In the serum-free and preimmune serum control wells, the typical pattern of nonagglutination was observed (cells uniformly spread on the well bottom) at all dilutions of the antiserum. This result implies that the polyclonal antibodies in the antiserum recognized antigenic determinants of HPF1 on the yeast cell surface and caused the agglutination.

Immunolocalization of HPF1 in the Cell Wall. The cells collected in late exponential phase were fixed and embedded in resin before being cut in ultrathin sections and examined by transmission electron microscopy after labeling with the anti-HPF1 antibodies and staining with gold labeled Protein A (Figure 1). HPF1 was mainly detected in the cell wall and occasionally within the cytoplasm or in vacuoles. The percentage of gold particles in the cell wall greatly outnumbered that in the vacuoles and in cytoplasm.

The labeled material was not evenly distributed through the cell wall. Gold particles were more concentrated on the periphery (outermost layer) of the cell wall or near the cytoplasmic membrane (innermost layer of the cell wall) whereas sparse labeling was detected within the cell wall itself (Figure 1). This result is in agreement with that of the agglutination assay which revealed the presence of HPF1 on the cell surface.

The labeling in the innermost layers of the cell wall was intense (Figure 1), consistently observed and may have corresponded to an accumulation of HPF1 in the periplasmic space (under the conditions used in this study, the periplasmic space could not be visualized separately from the innermost layers of the cell wall). Other immunological studies have similarly observed cell wall mannoproteins and secreted glycoproteins in the innermost layers of the cell wall (Elorza et al., 1993; Cailliez et al., 1992; Lu et al., 1994) and Pastor et al. (1984) observed cell wall mannoproteins in transit through the periplasmic space before reaching their destination on the outer surface. The labeling in the central part of the cell wall was sparse, more or less randomly spread and no preferential secretion pathways could be observed. HPF1 was therefore probably freely diffusing from the innermost part of the cell wall to its destination on the cell wall surface. Alternatively, these results could simply reflect the two different locations

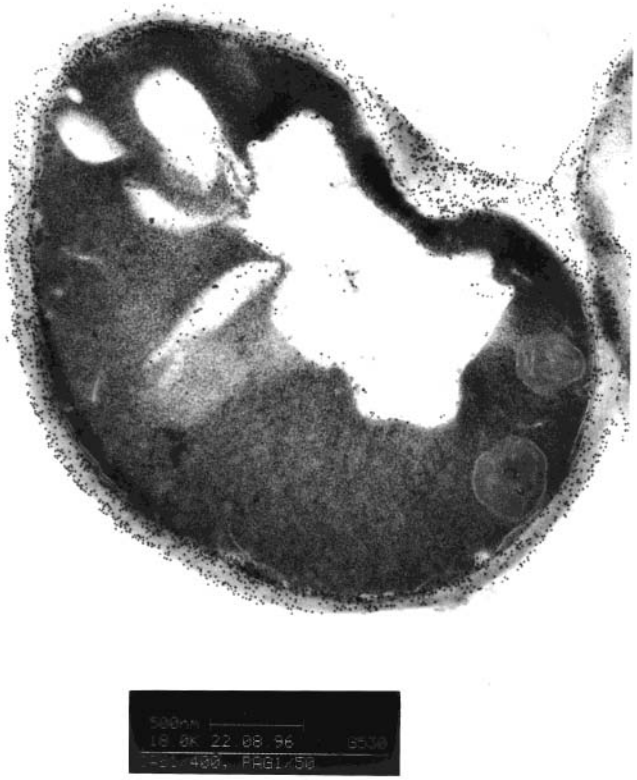

Figure 1. Site of HPF1 in *Saccharomyces cerevisiae* Maurivin PDM cells in late exponential phase**.** The binding of anti-HPF1 antibodies to the ultrathin sections of the cells were visualized as dense dots by binding of Protein A conjugated to colloidal gold to the yeast bound anti-HPF1 antibodies.

of the two antigenic determinants in the purified HPF1 solution used for the immunization.

The cytoplasmic labeling was not uniform but mainly concentrated on the periphery near the cytoplasmic membrane, as described above. During the cell wall mannoprotein biosynthesis the protein and mannan moieties are synthesized intracellularly and modified within the endoplasmic reticulum in the course of their migration to the cell wall (Scheckman and Novick, 1982). Thus, the anti-HPF1 antibodies might have recognized a non-glycosylated precursor form of HPF1 [or alternatively the mature form accumulated intracellularly as suggested by Cailliez et al. (1994) for other cell wall glycoproteins] in the cytoplasm before its extrusion in the extracytoplasmic medium. Immunological cytoplasmic detection of cell wall mannoproteins has been reported (Linnemans et al., 1977; Cailliez et al., 1992).

In some sections (not shown herein), gold particles were also detected in small vesicles which could correspond to secretory vesicles. However, the number of them was small. Some gold particles were also seen in the vacuoles as seen by others for other mannoproteins (Meyer and Matile, 1975; Horisberger and Vonlanthen, 1977; Linnemans et al., 1977). No labeling was observed in the nucleus (data not shown) or the mitochondria (see Figure 1).

CONCLUSIONS

The extraction of yeast haze-protective mannoprotein material (HPM) has been investigated. Methods which disrupt covalent bonds (DTE and Zymolyase treatment) or physically destroy cell components (French press, autoclave methods) resulted in the extraction of mannoprotein fractions which exhibited no or poor haze-protective ability. Extraction of HPM was facilitated by methods having a mild impact (disruption of the noncovalent bonds) on the cell envelope such as EDTA treatment or boiling in Tris buffer. Accordingly, HPM was thought to be noncovalently linked to other cell wall components and loosely associated with the cell wall.

Using the agglutination assay and the immunolabeling of sections examined by transmission electron microscopy, the presence of HPF1 on the cell wall surface was confirmed. HPF1 was also present in the inner layers of the cell wall and more sparsely in the central layers. The distribution of HPF1 in the wall was in agreement with that of other cell wall mannoproteins like α-agglutinins or secreted glycoproteins which also accumulated in the innermost parts of the wall before further migration to the outer surface (Pastor et al., 1984; Cailliez et al., 1992; Lu et al., 1994).

Given the yields of HPM extracted from the cells by the methods used in this research, plans to isolate large quantities of HPM from yeast cells are probably unrealistic. This is because the highest yield of total mannoproteins found here was 1.65 g per 100 g of wet cells. To reduce protein haze to 20% or less of the initial values (this would probably stabilize all but very protein-rich white wines), a concentration of approximately 500 mg/L of this crude mannoprotein material would be needed. Therefore, at least 300 kg of yeast cells (wet weight) would need to be processed to produce enough material to stabilize 10 000 L of wine.

HPF1 was previously purified from white (Waters et al., 1993) and red wine (Waters et al., 1994a). Some HPF1 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 in both cases, it is also possible that HPF1 was secreted during fermentation. The secretion of HPF1 and other haze-protective mannoproteins during alcoholic fermentation may prove to be a better source of HPM than subsequent extraction of HPM from the yeast cells. This was explored in a subsequent study (Dupin et al., 2000).

ABBREVIATIONS USED

BSA, bovine serum albumin; Con-A, Concanavalin-A; DTE, dithioerythritol; EDTA, ethylenediamine tetraacetate; HPF1, haze-protective factor 1 (a specific mannoprotein in the HPM group); HPF2, haze-protective factor 2 (a specific mannoprotein in the HPM group); HPM, haze-protective mannoprotein material; LEP, late exponential phase; SDS, sodium dodecyl sulfate; CDGJM, chemically defined grape juice medium; SP, stationary phase; PBS, phosphate-buffered saline.

ACKNOWLEDGMENT

We thank Holger Gockowiak of the Australian Wine Research Institute for help and guidance with the microbiological aspect of this work, Ray Wilkinsin from VetLab for the production of antibodies and Lyn Waterhouse, Centre for Electron Microscopy and Microstructure Analysis, The University of Adelaide, for her guidance in immunocytochemistry. Wine samples were donated by Orlando Wyndham Wines (Rowland Flat, SA, Australia).

LITERATURE CITED

Al-Mahmood, S.; Giummelly, P.; Bonaly, R. Structural modifications of mannans during flocculation of *Kluyveromyces bulgaricus*. *Appl. Microbiol. Biotechnol.* **1987**, *26*, 462−467.

Amri, M. A.; Bonaly, R.; Duteurtre, B.; Moll, M. Yeast flocculation: influence of nutritional factors on cell wall composition. *J. Gen. Microbiol.* **1982**, *128*, 2001−2009.

Arnold, W. N. Location of acid phosphatase and β-fructofuranosidase within yeast cell envelopes. *J. Bacteriol.* **1972**, *112*, 1346−1352.

Bellal, M.; Benallaoua, S.; Elfoul, L.; Bonaly, R. Différences structurales des peptidomannanes pariétaux chez le *Kluyveromyces lactis*, entre souches floculante et non floculante. *Can. J. Microbiol.* **1995**, *41*, 323−335.

Cailliez, J. C.; Poulain, D.; Mackenzie, D. W. R.; Polonelli, L. Cytological immnunodetection of yeast glycoprotein secretion. *Eur. J. Epidemiol.* **1992**, *8*, 452−459;

Cailliez, J. C.; Cantelli, C.; Séguy, N.; Conti, S.; Gerloni, M.; Morace, G.; Polonelli, L. Killer toxin secretion through the cell wall of the yeast *Pichia anomala*. *Mycopathologia* **1994**, *126*, 173−177.

De Nobel, J. G.; Djikers, C.; Hooijberg, E.; Klis, F. M. Increased cell wall porosity in *Saccharomyces cerevisiae* after treatment with dithiothreitol or EDTA. *J. Gen. Microbiol.* **1989**, *135*, 2077−2084.

De Nobel, J. G.; Klis, F. M.; Priem, J.; Munnik, T.; Van Den Ende, H. The glucanase-soluble mannoproteins limit cell wall porosity in *Saccharomyces cerevisiae*. *Yeast* **1990**, *6*, 491−499.

Dupin, I. Production and localisation of haze protective material from *Saccharomyces cerevisiae*. Ph.D. Thesis, University of Adelaide, South Australia, 1997.

Dupin, I. V. S.; McKinnon, B. M.; Ryan, C.; Boulay, M.; Markides, A. J.; Jones, G. P.; Williams, P. J.; Waters, E. J. *Saccharomyces cerevisiae* mannoproteins that protect wine from protein haze: Their release during fermentation and lees contact and mechanism of action. *J. Agric. Food Chem.* **2000**, submitted for publication.

Elorza, M. V.; Murgui, A.; Sentandreu, R. Dimorphism in *Candida albicans*: contribution of mannoproteins to the architecture of yeast and mycelial cell walls. *J. Gen. Microbiol.* **1985**, *131*, 2209−2216.

Esmon, B.; Novick, P.; Schekman, R. Compartmentalized assembly of oligosaccharides on exported glycoproteins in yeast. *Cell* **1981**, *25*, 451−460.

Ey, P. L.; Prowse, S. J.; Jenkin, C. R. Isolation of pure IgG_1, IgG_{2a}, IgG_{2b} immunoglobulins from mouse serum using protein A sepahrose. *Biochemistry* **1978**, *15*, 429−436.

Fleet, G. H. Cell walls. In *The yeasts. Volume 4. Yeasts organelles*; Rose, A. H., Harrison, J. S., Eds; Academic Press: London, 1991.

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

Horisberger, M.; Vonlanthen, M. Location of mannan and chitin on thin sections of budding yeasts with gold markers. *Arch. Microbiol.* **1977**, *115*, 1−7.

Horvath, A.; Riezman, H. Rapid protein extraction from *Saccharomyces cerevisiae*. *Yeast* **1994**, *10*, 1305−1310.

Janson, J. C.; Ryden, L. *Protein purification. Principles, high-resolution methods, and applications*; Janson, J. C., Ed.; VCH Publishers: New York, 1989.

Katohda, S.; Abe, N.; Matsui, M.; Hayashibe, M. Polysaccharide composition of the cell wall of baker's yeast with special reference to cell walls obtained from large- and small-sized cells. *Plant Cell Physiol.* **1976**, *17*, 909−919.

Ledoux, V.; Dulau, L.; Dubourdieu, D. Interprétation de l'amélioration de la stabilité protéique des vins au cours de l'évelage sur lies. *J. Inter. Sci. Vigne Vin.* **1992**, *26*, 239−251.

Linnemans, W. A. M.; Boer, P.; Elbers, P. F. Localization of acid phosphatase in *Saccharomyces cerevisiae*: a clue to cell wall formation. *J. Bacteriol.* **1977**, *131*, 638−644.

Lu, C. F.; Kurjan, J.; Lipke, P. N. A pathway for cell wall anchorage of *Saccharomyces cerevisiae* α-agglutinin. *Mol. Cell. Biol.* **1994**, *14*, 4825−4833.

MacKinnon, B. A study of protein haze prevention in model wine systems. B.Ag.Sci. Honours thesis, University of Adelaide, South Australia, 1996.

Meyer, J.; Matile, P. Subcellular distribution of yeast invertase isoenzymes. *Arch. Mikrobiol.* **1975**, *103*, 51−55.

Miller, G. C.; Amon, J. M.; Gibson, R. L.; Simpson, R. F. Loss of wine aroma attributable to protein stabilization with bentonite or ultrafiltration. *Aust. Grapegrower Winemaker* **1985**, *256*, 46−50.

Moine-Ledoux, V.; Dubourdieu, D. Interprétation moléculaire de l'amélioration de la stabilité protéique des vins blancs au cours de leur élevage sur lies. *Rev. Oenol.* **1998**, *86*, 11−14.

Moine-Ledoux, V.; Dubourdieu, D. An invertase fragment responsible for improving the protein stability of dry white wines. *J. Sci. Food Agric.* **1999**, *79*, 537−543.

Molloy, C.; Shepherd, M. G.; Sullivan, P. A. Identification of envelope proteins of *Candida albicans* by vectorial iodination. *Microbios* **1989**, *57*, 73−83.

Ouchterlony, Ö. Antigen−antibody reactions in gels. *Ark. Kemi. Mineral Geol.* **1949**, *26*, 1.

Paetzold, M.; Dulau, L.; Dubourdieu, D. Fractionnement et caractérisation des glycoprotéines dans les moûts de raisins blancs. *J. Int. Sci. Vigne Vin*. **1990**, *24*, 13−28.

Pastor, F. I. J.; Valentin, E.; Herrero, E.; Sentandreu, R. Structure of the *Saccharomyces cerevisiae* wall. Mannoproteins released by Zymolyase and their contribution to wall architecture. *Biochim. Biophys. Acta*. **1984**, *802*, 292−300.

Peat, S.; Whelan, W. J.; Edwards, T. E. Polysaccharides of Baker's yeast. Part IV. Mannan. *J. Chem Soc.* **1961**, p 29.

Pellerin, P.; Waters, E. J. Brillouet, J−M.; Moutounet, M. Effet de polysaccharides sur la formation de trouble protéique dans un vin blanc. *J. Int. Sci. Vigne Vin*. **1994**, *28*, 23−225.

Puigdeu, M.; Lopeztamames, E.; Buxaderas, S.; Torreboronat, M. C. Influence of must racking and fining procedures on the composition of white wine. *Vitis* **1996**, *35*, 141−145.

Saulnier, L.; Mercereau, T.; Vezhinet, F. Mannoproteins from flocculating and nonflocculating *Saccharomyces cerevisiae* yeasts. *J. Sci. Food Agric.* **1991**, *54*, 275−286.

Schekman, R.; Novick, P. The secretory process and yeast cell-surface assembly. In *The molecular biology of the yeast Saccharomyces*; Strathern, J. N., Jones, E. W., Broach, J. R., Eds.; Cold Spring Harbor Laboratory: Cold Spring Harbor, 1982.

Schwartzberg, H. G.; Hartel, R. W. *Physical chemistry of foods*; Marcel Dekker: New York, 1992.

So, L.; Goldstein, I. J. The interaction of concanavalin A with α-mannans from a variety of microorganisms. *J. Biol. Chem.* **1968**, *243*, 2003−2007.

Trimble, R. B.; Maley, F. Subunit structure of external invertase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **1977**, *252*, 4409−4412.

Valentin, E.; Herrero, E.; Pastor, F. I. J.; Sentandreu, R. Solubilization and analysis of mannoprotein molecules from the cell wall of *Saccharomyces cerevisiae*. *J. Gen. Microbiol.* **1984**, *130*, 1419−1428.

Valentin, E.; Herrero, E.; Rico, H.; Miragall, F.; Sentandreu, R. Cell wall mannoproteins during the population growth phases in *Saccharomyces cerevisiae*. *Arch. Microbiol.* **1987**, *148*, 88−94.

Voilley, A.; Lamer, C.; Dubois, P.; Feuillat, M. Influence of macromolecules and treatments on the behavior of aroma compounds in a model wine. *J. Agric. Food Chem.* **1990**, *38*, 248−251.

Waters, E. J.; Wallace, W.; Williams, P. J. Heat haze characteristics of fractionated wine proteins. *Am. J. Enol. Vitic.* **1991**, *42*, 123−127.

Waters, E. J.; Wallace, W.; Williams, P. J. Identification of heat-unstable wine proteins and their resistance to peptidases. *J. Agric. Food Chem.* **1992**, *40*, 1514−1519.

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

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

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

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

Received for review May 14, 1999. Revised manuscript received December 23, 1999. Accepted December 30, 1999. I.V.S.D. was supported by an Overseas Postgraduate Research Scholarship from the University of Adelaide. Financial support was provided by the Grape and Wine Research and Development Corp.

JF9905020