Annexe 3.2 :

Annex 3.2 :

Mannoprotéines et vins : teneurs naturelles et relargage au cours de la fermentation

Mannoproteins and wine : natural content and release during fermentation

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J. Sci. Food Agric. 1987, **41**, 277–286

Exocellular Polysaccharides from *Saccharomyces* in Wine

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(Received 2 March 1987; revised version received 21 April 1987; accepted 28 April 1987)

ABSTRACT

The exocellular polysaccharides released by commercial dried Saccharomyces *yeasts in wine and fermented media have been studied during alcoholic fermentation and storage over lees for several months. Hexadecyltrimethylammonium bromide (Cetavlon) precipitation and affinity chromatography (Con A–Sepharose) were used to separated a mannoprotein from an unpurified macromolecular fraction containing glucose, mannose and protein. The mannoprotein is the major component of the yeast exocellular polysaccharides.*

The soluble polysaccharide content, by h.p.l.c., depends on the yeast strain, the fermentation temperature and the duration of storage over yeast lees. In white wine production, the contact of wine with yeast lees and the stirring of lees increase its soluble polysaccharide content.

Key words: Yeast exocellular polysaccharides, mannoprotein, glucan, white wine production, lees.

1 INTRODUCTION

In the past ten years, important progress has been made in understanding the yeast cell envelopes of *Saccharomyces cerevisiae*. In particular the molecular structures of the different cell wall components such as mannoproteins or glucans

J. Sci. Food Agric. 0022-5142/87/$03.50 © Society of Chemical Industry, 1987. Printed in Great Britain

have been partially defined. Several reviews dealing with this subject have appeared in recent years,[1,2] but few results have been published on the structure of polysaccharides released by the yeast into the fermentation medium. Although these macromolecules are present in concentrations of several hundred milligrams per litre, they have been studied by few authors. Usseglio-Tomasset[3] found approximately 80% mannose and 20% glucose in the exocellular polysaccharides released by *S. cerevisiae* during the fermentation of a synthetic medium. Wucherpfennig *et al.*[4] also reported the presence of mannose (70%) and glucose (30%) in the hydrolysates of yeast polysaccharides. These results revealed the importance of mannose in the composition of wine polysaccharides. Villettaz *et al.*[5] isolated (from a rosé wine fermented by *S. uvarum*) an α-D-mannan by precipitation with Fehling's reagent. They identified this macromolecule as the mannan of the yeast cell wall. Hitherto no purification of an exocellular yeast glucan has been described.

This paper reports a fractionation of exocellular polysaccharides from different yeast strains. The release of these polysaccharides in synthetic medium and in wine has been followed during alcoholic fermentation and storage over yeast lees, and the influence of various factors has been analysed.

2 EXPERIMENTAL

2.1 Fermentation medium

The synthetic medium of the Institut d'Oenologie de Bordeaux[6] was used. Free of polysaccharides, this medium contained sugars, organic acids, mineral salts, amino acids and vitamins at the same concentrations as those found in wine. Its composition was: glucose 85 g litre^{-1}; fructose 85 g litre^{-1}; L-tartaric acid 3 g litre^{-1}; DL-malic acid 6 g litre^{-1}; citric acid 0·3 g litre^{-1}; asparagine 2 g litre^{-1}; potassium phosphate 2 g litre^{-1}; ammonium sulphate 2 g litre^{-1}; magnesium sulphate 0·2 g litre^{-1}; manganese sulphate 0·01 g litre^{-1}; meso-inositol 0·3 g litre^{-1}. The pH was adjusted to 3·2. After autoclaving for 10 min at 105°C, 10 ml of a sterile vitamin solution (biotin 4 mg litre^{-1}; thiamin 100 mg litre^{-1}; pyridoxin 100 mg litre^{-1}; nicotinic acid 100 mg litre^{-1}; pantothenic acid 100 mg litre^{-1}) was added per litre of synthetic medium.

2.2 Yeast strains

The following commercial dried yeasts were used: *S. cerevisiae* 'Fermivin' (Gistbrocades, Seclin, France), 'Lévactif 3' (Siha–Getränkeschutz, Langenlonsheim, West Germany), and 'Uvaferm CS 2', 'Uvaferm CEG' and 'Uvaferm CM' (three strains from Novo Ferment, Basel, Switzerland); and *S. bayanus* 'Actiflore 1 SB' (Setric Biologie, Colomiers, France). The medium was inoculated with 100 mg litre^{-1} of dry yeasts. Samples were taken during the alcoholic fermentation and during storage over lees (both at 20°C).

2.3 Determination of total polysaccharides

The polysaccharide content of centrifuged media was determined by size exclu-

sion using high-performance liquid chromatography (h.p.l.c.). This method does not require any preliminary purification of the macromolecules.[7]

2.4 Separation and purification of glycosidic macromolecules

Fermentation media (500 ml) centrifuged at 7500×*g* for 20 min were concentrated (150 ml) by ultrafiltration (Sartorius SM 165 25, Sartorius-France, Palaiseau, France) and dialysed against distilled water. The ultrafiltration was conducted at a pressure of 0·1 or 0·2 MPa; the membrane cut-off was MW 10 000. The total exocellular polysaccharides could also be isolated from culture media by ethanol precipitation (five parts ethanol to one part medium). These two methods gave similar results. The glycosidic macromolecules were then separated by

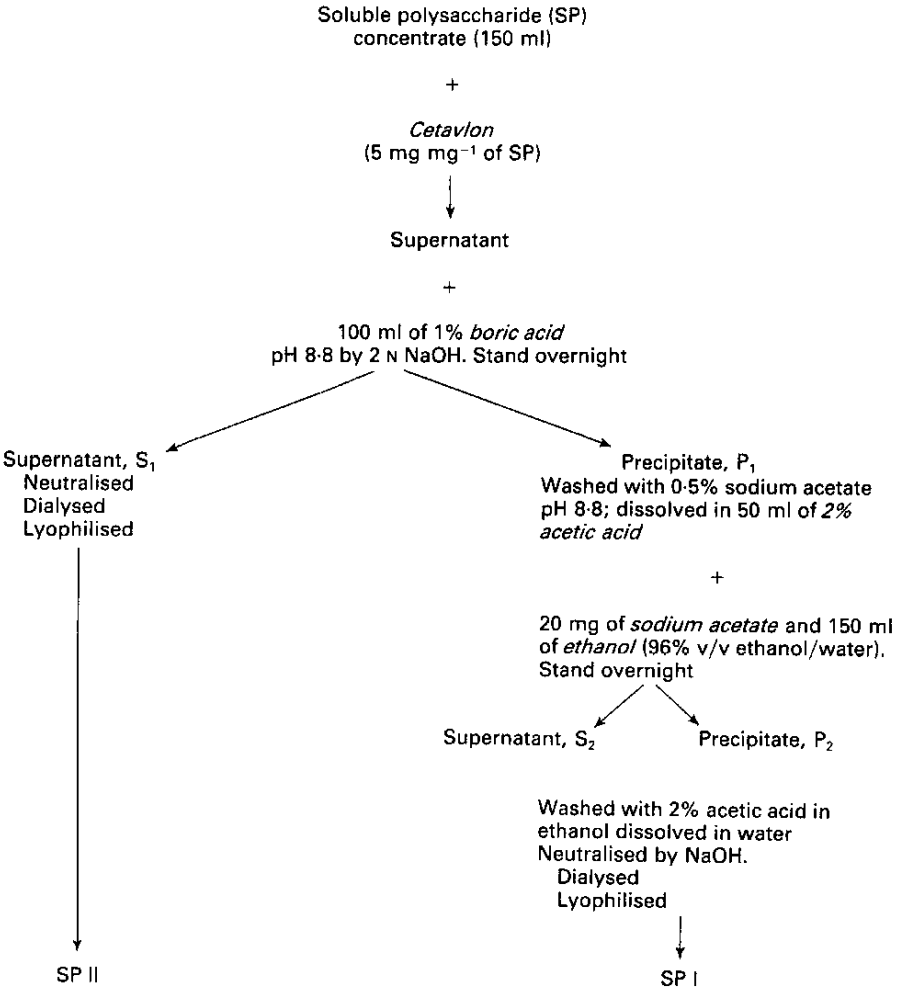

Fig. 1. Separation of yeast polysaccharides by hexadecyltrimethylammonium bromide (Cetavlon).

hexadecyltrimethylammonium bromide (Cetavlon) fractionation according to Nakajima and Ballou.[8] The separation scheme is given in Fig. 1.

The interaction of microbial α-mannans with concanavalin A has been studied by So and Goldstein.[9] This method was used in the present work to fractionate the exocellular yeast polysaccharides. Yeast exocellular macromolecules (*c.* 7 mg) dissolved in elution buffer (0·1 M sodium acetate, pH 5·0; 0·2 M sodium chloride) were passed through a concanavalin A–Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) column (0·9×13 cm), and 3-ml fractions were collected. Optical density at 280 nm was followed. Unbound macromolecules were in peak A; bound macromolecules were then eluted with an α-D-methyl mannoside solution 0·2 M in the starting buffer (peak B) (Fig. 2). Fractions corresponding to each peak were bulked. The peak B bulk was dialysed, and desalted on Sephadex

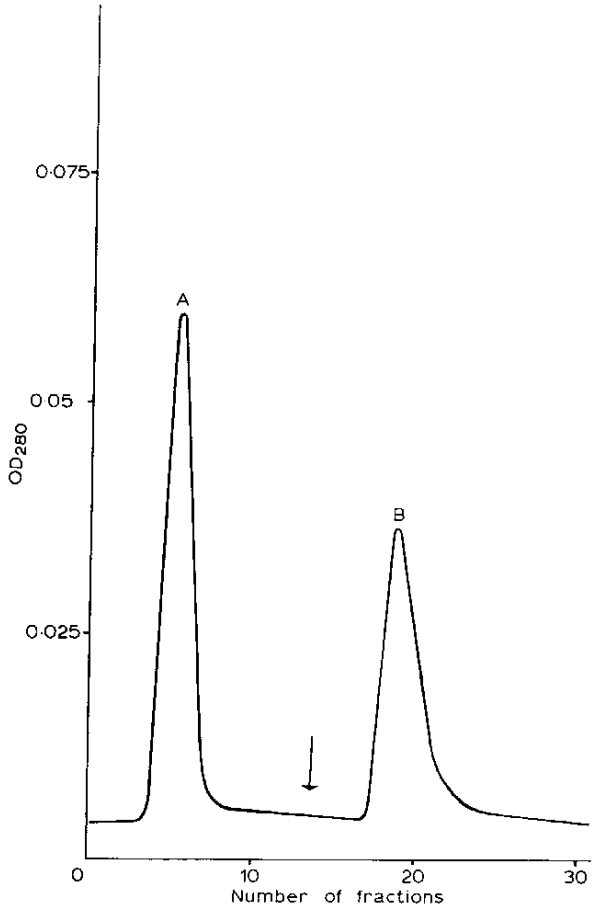

Fig. 2. Affinity chromatography of exocellular yeast polysaccharides ('Uvaferm CM') on Con A–Sepharose. The column was eluted with 0·1 M sodium acetate (pH 5·0), 0·2 M sodium chloride buffer. At the point indicated by the arrow, elution was started with 0·2 M α-D-methyl mannoside dissolved in the same solution. Optical density at 280 nm (OD_{280} full scale: 0·1) was followed.

G-25 gel (Pharmacia Fine Chemicals). The carbohydrate contents of peaks A and B were determined by the phenol–sulphuric acid method of Dubois *et al.*[10] and the protein contents were measured according to Lowry *et al.*[11] The polysaccharide composition of peaks A and B was determined by gas chromatography as described below.

2.5 Analysis of polysaccharides by gas chromatography

Fractions (*c.* 200 μg) of lyophilised polysaccharides with meso-inositol (internal standard 100 μg) were hydrolysed by 4 M trifluoroacetic acid at 100°C for 4 h (Fournet, B., personal communication) in Sovirel tubes. The acid was then evaporated under vacuum at 40°C. The monomers of the hydrolysed polysaccharides were then trimethylsilylated[12] and analysed on a capillary column CP Sil 5, 25 m in length, under a hydrogen pressure of 75 kPa. The temperature was programmed from 80 to 260°C by 2°C min^{-1} (Intersmat IGC 12).

3 RESULTS AND DISCUSSION

3.1 Fractionation of exocellular polysaccharides of *Saccharomyces* yeasts

3.1.1 Composition of total exocellular polysaccharides

Table 1 shows the composition of exocellular polysaccharides released by different yeast strains. The proportion of mannose and glucose depends on the yeast strain studied and the age of the culture. The major monosaccharide found was always mannose, and the glucose content decreased with the age of the cultures.

TABLE 1
Percentage Composition of Exocellular Polysaccharides Released by Different Commercial *Saccharomyces* Yeasts

Yeast strain	*Culture age (days)*	*Mannose (%)*	*Glucose (%)*	*SP (mg litre^{-1})*
Uvaferm CM	40	72	28	198
	140	90	10	220
Actiflore 1 SB	40	89	11	290
	140	90	10	308
Uvaferm CEG	40	88	12	274
Uvaferm CS 2	40	76	24	225

3.1.2 Fractionation of exocellular polysaccharides

Cetavlon precipitation of yeast polysaccharides as their borate complexes allowed the isolation of two macromolecular fractions SP I and SP II (Fig. 1). Protein and sugar contents of each fraction were determined by the Lowry *et al.* method[11] and phenol–sulphuric acid measurement,[10] respectively (Table 2). The monosaccharide composition of fractions SP I and SP II are given in Table 3. Fraction SP I contained roughly 10% protein; mannose was the only sugar found in the glycan

TABLE 2
Polysaccharide (polys.) and Protein (prot.) Percentages of Soluble Polysaccharide Fractions SP I and SP II

Yeast strain	*Culture age (days)*	*SP I*		*SP II*	
		polys. (%)	*prot. (%)*	*polys. (%)*	*prot. (%)*
Uvaferm CM	40	84	16	38	62
	140	92	8	28	72
Uvaferm CEG	40	87	13	27	73
	140	80	20	26	74
Uvaferm CS 2	40	91	9	60	40
	90	90	10	62	38
Actiflore 1 SB	140	92	8	39	61

TABLE 3
Percentage Composition of Soluble Polysaccharide Fractions SP I and SP II

Yeast strain	*Culture age (days)*	*SP I*		*SP II*	
		Mannose (%)	*Glucose (%)*	*Mannose (%)*	*Glucose (%)*
Uvaferm CM	40	96	4	54	46
Uvaferm	140	100	—	40	60
Uvaferm CS 2	40	100	—	28	72
Uvaferm	90	97	3	29	70
Uvaferm CEG	140	100	—	45	55
Actiflore 1 SB	140	100	—	48	52

TABLE 4
Polysaccharide (polys.), Protein (prot.) Contents and Percentage Composition of Soluble Polysaccharide Fractions A and B of the Yeast 'Uvaferm CM'

A		*B*		*A*		*B*	
polys. (%)	*prot. (%)*	*polys. (%)*	*prot. (%)*	*Mannose (%)*	*Glucose (%)*	*Mannose (%)*	*Glucose (%)*
44	56	88	12	49	51	96	4

moiety. Fraction SP II contained approximately 50% protein; its glycan moiety was composed of 50% each of glucose and mannose.

Concanavalin A–Sepharose 4B chromatography carried out on one strain of *Saccharomyces* ('Uvaferm CM') confirmed the results obtained by the Cetavlon method. The protein and sugar contents and monomer compositions of the two molecular fractions (A and B) (Fig. 2) are given in Table 4. Fraction B compared with fraction SP I and fraction A with fraction SP II.

3.2 Effects of yeast strain and duration of ageing on yeast polysaccharide release in fermentation medium

The rapid method for determination of polysaccharides by h.p.l.c. (section 2.3) has enabled us to follow easily the evolution of soluble polysaccharides (released

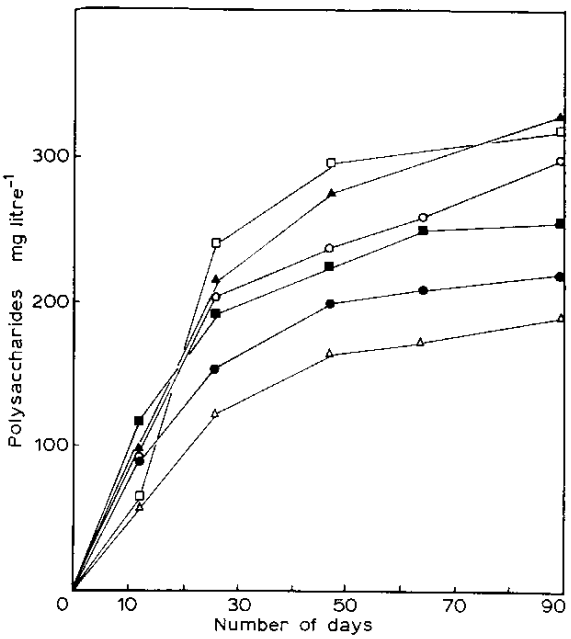

Fig. 3. Yeast polysaccharides released in a synthetic solution by commercial strains. ▲ 'Uvaferm CEG'; □ 'Actiflore 1 SB'; ○ 'Lévactif 3', ■ 'Uvaferm CS 2'; ● 'Uvaferm CM'; △ 'Fermivin'.

by the six yeast strains) during the alcoholic fermentation and storage over lees.

Figure 3 shows the increases obtained on a synthetic medium at 20°C. For a given strain, for example 'Uvaferm CEG', the yeast biomass released 180 mg litre^{-1} of polysaccharides into the medium during the alcoholic fermentation. This enrichment in macromolecules continued after the end of the fermentation. Two months after inoculation, the fermented medium contained about 280 mg litre^{-1} of yeast polysaccharides and 330 mg litre^{-1} after a further month. The contents of exocellular polysaccharides in fermented media varied according to the yeast strain studied. In the present work the amounts of polysaccharides released during the alcoholic fermentation increased from 100 to 240 mg litre^{-1}; three months after inoculation, the polysaccharide content was between 190 and 330 mg litre^{-1}. For a given strain the temperature did not influence the polysaccharide released during the first 10 days of the alcoholic fermentation. However, temperature became important towards the end of the fermentation and even more so during yeast–lees contact. In fact one month after inoculation of a synthetic medium the polysaccharide content in the samples kept at 22°C and 35°C differed by a factor of two (Fig. 4).

3.3 Production of exocellular yeast polysaccharides during ageing on lees of a Bordeaux white wine

This trial was conducted during the 1985 harvest on wines made from Semillon grapes picked in the Graves region. From a tank of must racked off from its

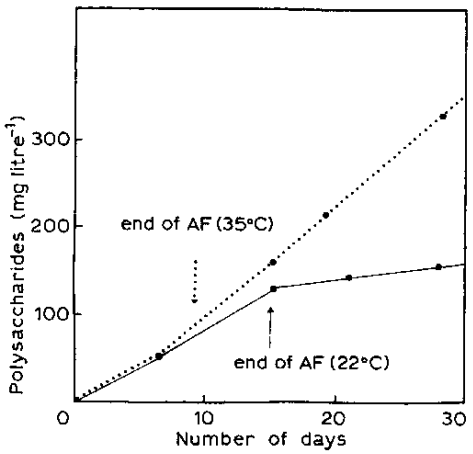

Fig. 4. Effect of temperature on yeast polysaccharide release (·····35°C; ——22°C, 'Uvaferm CM'). AF=Alcoholic fermentation.

sediment and then inoculated with dry yeast 'Uvaferm CS 2' four lots were generated:

— lot 1 (control): fermented in a 100 hl vat at 18°C, racked off at the end of the alcoholic fermentation and vat-aged on light lees
— lot 2: fermented in new oak barrels without temperature control; racked off at the end of the alcoholic fermentation and barrel aged on light lees
— lot 3: barrel fermented and aged on the total volume of fermentation lees (without racking at the end of the alcoholic fermentation)

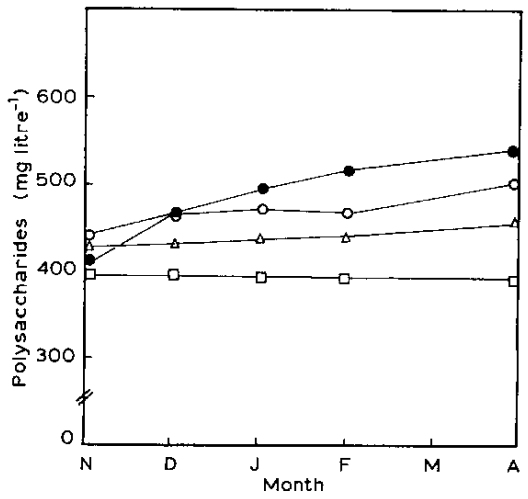

Fig. 5. Exocellular yeast polysaccharides released into white wine ageing on lees. □ Lot 1: tank aged on light lees; △ lot 2: barrel aged on light lees; ○ lot 3: barrel aged on fermentation lees; ● lot 4: barrel aged on fermentation lees periodically resuspended.

— lot 4: the same conditions of fermentation and racking as for lot 3 but the lees were resuspended during barrel ageing every 15 days

Samples were taken during storage over lees for six months (November through April). The polysaccharide content, determined by h.p.l.c. (Fig. 5), remained constant during ageing on light lees in lot 1; the same result was obtained with lot 2 but with a higher polysaccharide content. An even higher polysaccharide level was found in wine aged on the total volume of fermentation lees in lots 3 and 4. After six months of ageing on lees, the polysaccharide contents of lots 2, 3 and 4 were respectively 17, 28 and 38% higher than that of lot 1.

Yeast–lees contact was responsible for an increase in soluble polysaccharide content in wine. This increase is greater when the contact is with the total volume of fermentation lees (i.e. without racking after the alcoholic fermentation) and even larger when the lees are periodically resuspended. Some preliminary tastings at the Institut d'Oenologie indicated that there could be a positive effect on perceptible roundness and fullness of wine flavour related to increased total polysaccharide content.

4 CONCLUSIONS

Mannan was a major exocellular polysaccharide component of all commercial yeasts studied. On careful isolation (Cetavlon precipitation) yeast mannans seemed covalently linked to protein. Similar results were obtained after affinity chromatography. The term mannoprotein more correctly describes their macromolecular status. Structural studies will be carried out on the mannoprotein to confirm the present results. The second macromolecular component (glucan–mannan–protein) needs further purification. The high protein percentage might be explained by a co-precipitation of proteins and polysaccharides.

The release of yeast exocellular polysaccharides in the fermentation medium varied with yeast strain, temperature and duration of storage over lees. During the alcoholic fermentation this release could be associated with cell wall synthesis. In living cells mannoproteins are mainly synthesised in the cytoplasm.[13] The macromolecules might not be entirely used in the cell wall structure and a portion might be solubilised in the fermentation medium. During contact with the lees cell wall autolysis occurs as a result of the action of several enzymes[14] (cell wall glucanase might play an important role) and soluble mannoproteins might then be released.

The release of yeast polysaccharides has also been found during yeast–lees contact in white wine production. The stirring of the lees during wine ageing in barrels seems to increase the soluble yeast polysaccharide content to an even greater extent. In tasting trials, these wines are often preferred.

ACKNOWLEDGEMENT

The authors thank Professor A. Bertrand for his help with the analysis of polysaccharides by gas chromatography.

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Research Note

Evolution of Grape (Carignan noir cv.) and Yeast Polysaccharides During Fermentation and Post-Maceration

T. DOCO[1]*, J.-M. BRILLOUET[2], and M. MOUTOUNET[3]

Soluble polysaccharides (yeast mannoproteins, grape type II arabinogalactans, and rhamnogalacturonan II) increased in concentration during fermentation of must to wine (9 days), then stabilized during the post-maceration (final time 26 days). Soluble proteins exhibited an opposite behavior.

KEY WORDS: wine polysaccharides, mannoproteins, type II arabinogalactans, rhamnogalacturonan II, proteins.

Wine polysaccharides are now better understood (3,6,9,12,17,21), and some of them play a detrimental role in wine technology, *e.g.*, by fouling filtration membranes. Fractionation of wine polysaccharides by high performance size exclusion chromatography (HPSEC) was developed to study their molecular weight distributions (8,10,16). Purpose of this work was to study the changes in Carignan noir grape and yeast polysaccharides during fermentation and post-maceration.

Material and Methods

Plant material: Sound mature grapes (Carignan noir cv.) were obtained from INRA Pech-Rouge/Narbonne Experimental Station (Gruissan, France) in September 1992. Grapes were destemmed, crushed and, after addition of yeast (Fermivin, Gist Brocades, 2 g/100 L), allowed to ferment for nine days. Fermentation was followed by post-maceration (17 days). Samples (300 mL) were withdrawn during fermentation and post-maceration and immediately frozen at -20°C.

Decolorization of colloids: After thawing, 10-mL samples were depigmented by chromatography onto a column (4 × 2.5 cm) of Polyamide CC6 (Macherey-Nagel, Düren, Germany) previously washed with 1 *M* NaCl and equilibrated with 0.2 *M* NaCl. Colloidal material (polysaccharides, proteins, and residual polyphenols) eluting at the void volume, were collected, dialyzed extensively against distilled water (cut off: 1200 d), concentrated, and freeze-dried.

High performance size exclusion chromatography (HPSEC): Molecular weight distribution of the polysaccharides was determined by high performance size exclusion chromatography (HPSEC) (16). A Waters (Millipore, USA) M 510 solvent delivery system was connected to two serial Shodex OHpak KB-803 and KB-805 columns (30 × 0.8 cm, Showa Denkko, Japan) equilibrated at 1 mL/min with 0.1 *M* $LiNO_3$. An Erma-ERC 7512 (Erma, Japan) refractive index detector was used to monitor the elution profile in combination with Waters Baseline 810 software. Calibration was performed with narrow pullulan molecular-weight standards (9).

General: Polysaccharides were hydrolyzed with 2 *M* trifluoroacetic acid according to Albersheim *et al.* (1) and liberated monosaccharides were analyzed by gas-liquid chromatography of their alditol acetates (11). A Hewlett-Packard Model 5890 gas chromatograph fitted with a fused-silica capillary column (30 m × 0.32 mm *i.d.* bonded with OV 225 (DB-225, J & W Scientific; 0.25 μm film thickness) was used and operating parameters were described by Saulnier *et al.* (18). Total uronic acids were assayed according to Blumenkrantz and Asboe-Hansen (4). Protein contents were measured by the method of Lowry *et al.* (13) with bovine serum albumin as standard.

Results and Discussion

Evolution of depigmented wine colloids and their constituents during the fermentation-post-maceration is shown in Figure 1. Concentration of colloids increased progressively by 40% between zero time and 26 days reaching ~2 g/L. Both neutral and acidic polysaccharides were doubled during this period (neutral, 302 → 606 mg/L; acidic, 184 → 351 mg/L) reaching a quasi-plateau by nine days (*i.e.*, at the end of the fermentation period). Proteins behaved in the opposite way; their concentration decreased from 226 to 79 mg/L during fermentation, then stabilized until the end. The evolution of polysaccharides can be interpreted, in addition to the liberation of yeast mannoproteins during the fermentation, as the progressive release of carbohydrate polymers from skin and pulp cell walls. This interpretation is well sustained by the fact that white wines (no pomace contact during fermentation) exhibit roughly half of the amount of polysaccharides compared to red wines (2,19).

The lowering of proteins might be due to partial utilization by yeast and possibly to progressive insolubilization by phenolics (14). Formation of a tannin-protein insoluble complex is a well-known phenomenon in wine technology: *e.g.*, spontaneous precipitation of

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Acknowledgment: The authors are grateful to Mr. J.-L. Escudier (INRA, Station Expérimentale de Pech Rouge/Narbonne, Gruissan, France) for supplying wine samples.

Manuscript submitted for publication 20 January 1995.

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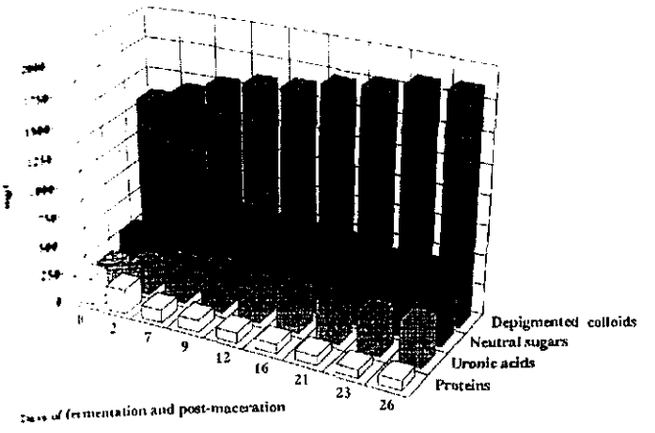

Fig. 1 Evolution of depigmented wine colloids, neutral and polysaccharides and proteins during the fermentation-post-maceration.

gelatine fining. Thus, post-maceration performed to enhance wine color did not, in our study, bring in any additional polysaccharides and proteins after completion of the fermentation. Final levels of these two components were in agreement with previously published data (2,3,6,15).

Monosaccharide compositions of wine depigmented colloids is given in Table 1.

Arabinose, galactose, mannose, and uronic acids (mainly galacturonic acid) were the major constituting sugars, and minor quantities of rhamnose, fucose, xylose, and glucose were found. Rare sugars (*i.e*, 2-O-methyl fucose, 2-O-methyl xylose, and apiose) which are specific constituents of RG-II (7,9) were also detected. All sugars but glucose and xylose increased from pressing to the end of the fermentation period (9 days), then remained fairly stable up to 26 days. Whereas most sugars were doubled, mannose increased tremendously from 28 to 162 mg/L due to the release of yeast mannoproteins. The (arabinose/galactose) ratio increased from 1 at zero time to an average of 1.6 between 9 and 26 days. Thus fermentation with presence of pomace is characterized by a progressive enrichment of the liquid phase by arabinose-rich polymers deriving from the cells walls. In fact, arabinose and galactose were found in a ratio close to 1 in musts from various grape cultivars (5) which agreed well with our figure at zero time.

Molecular weight distribution of depigmented colloids along fermentation and post-maceration was studied by HPSEC, and the refractometric profile is presented in Figure 2.

According to above statements about stability of wine colloids after the end of fermentation, chromatograms of 9- and 26-day samples were almost superimposable showing no evolution during this period. Population eluting between 13 and 15 minutes was absent at zero time and progressively enriched until the end of fermentation period; it corresponds to high molecular weight mannoproteins which form the bulk of yeast released polymers (12,20); however, their distribution extended to low molecular weight fragments.

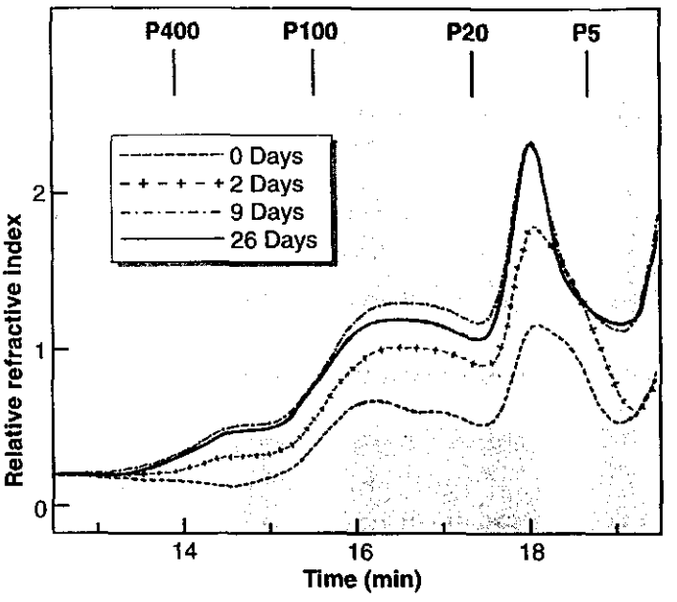

Fig. 2. Compared HPSEC profiles of colloids after 0 days, 2 days, 9 days, and 26 days. Eluates were monitored by differential refractometry. Elution times of pullanan standards (P5 → P400) are also shown.

A second heterogeneous population eluting between 15 and 17.5 minutes was also enriched during the fermentation period. According to previously published data, it must correspond to a complex mixture of neutral and weakly acidic type II arabinogalactans from grape (6,17). Indeed, this type of polymer is known to be present in grape must (5) (*i.e*., liquid phase at zero time), and they are also released from the cell wall structures during fermentation (6,17,21).

Table 1. Carbohydrate compositions of depigmented colloids[a].

Sample	2-O-MeFuc[c]	Rha[c]	Fuc[c]	2-O-MeXyl[c]	Ara[c]	Xyl[c]	Api[c]	Man[c]	Gal[c]	Glc[c]	U[d]
0[b]	1.7	30	2.7	1.4	92	17	2.0	28	93	34	184
2	3.4	55	4.4	2.3	169	15	3.4	84	135	28	253
7	3.7	55	3.6	1.9	177	14	1.8	116	114	29	313
9	4.9	79	5.8	2.7	232	14	4.3	148	147	29	365
12	4.2	75	5.8	2.3	211	14	3.6	162	162	31	297
16	4.7	75	5.4	2.6	226	16	4.0	144	130	30	335
21	3.9	64	5.0	2.5	229	15	4.4	148	140	30	339
23	3.5	61	3.9	2.0	197	26	3.6	142	119	32	333
26	3.6	62	3.6	2.1	204	13	3.6	156	127	31	351

[a]mg/L. [b]days of wine making. [c]Neutral sugars determined by gas-liquid chromatography and expressed as anhydrosugars; 2-O-MeFuc = 2-O-methyl fucose, Rha = rhamnose, Fuc = fucose, 2-O-MeXyl = 2-O-methyl xylose, Ara = arabinose, Xyl = xylose, Api = apiose, Man = mannose, Gal = galactose, Glc = glucose. [d]Uronic acids measured by the m-phenylphenol method and expressed as anhydrosugars.

Between 17.5 and 19 minutes, the must (0 day) exhibited a low molecular weight heterogeneous population (major peak at 18 minutes followed by a shoulder), and when fermentation proceeded, the shouldering fraction disappeared concomitantly with an increase of more symmetrical peak at 18 minutes. As already shown in a red wine (9), this peak must be mainly constituted of a rhamnogalacturonan II (RG-II), a complex pectic fragment from grape cell walls. Since RG-II specific sugars (*i.e.*, 2-O-methyl fucose, 2-O-methyl xylose, and apiose) were present at zero time, it possible that a RG-II-like structure pre-exists in must (main peak at 18 min.) and that during the fermentation period, more RG-II was released from insoluble cell walls.

Conclusions

HPSEC of depigmented colloids from a grape fermentation and post-maceration medium allowed the following of qualitative and quantitative changes in their molecular weight distribution. Passing from must to wine is characterized by a doubling of soluble polysaccharide concentration, a progressive appearance of yeast mannoproteins, and enrichment type II arabinogalactans and rhamnogalacturonan II. However, the apparent simplicity of HPSEC chromatograms hides a great complexity with regards to polysaccharides of which only few have been structurally studied.

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ŒNOLOGIE

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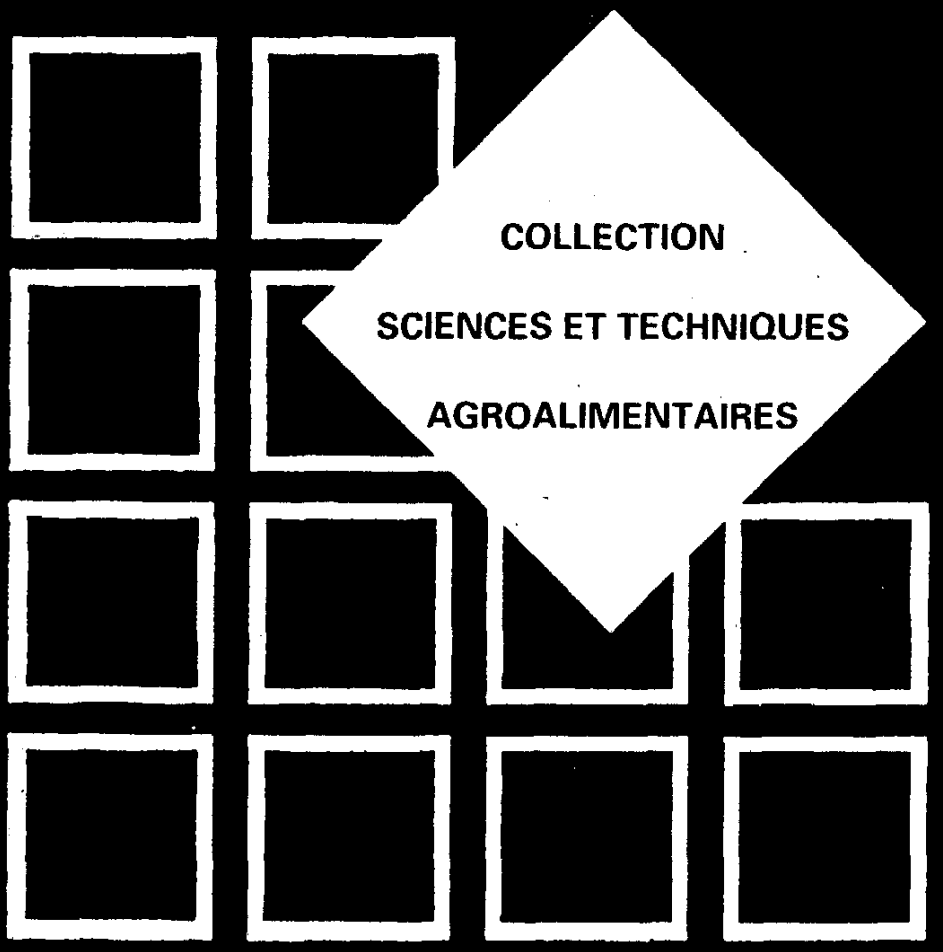

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correspondant(s) n'a pas encore été rapportée. La présence de xylogalacturonane dans les vins est donc une possibilité qui ne peut être écartée même si elle attend encore confirmation.

Autres Polysaccharides du raisin

Tous les polyosides du raisin identifiés à ce jour dans les moûts ou les vins appartiennent au groupe des polysaccharides pectiques. Pourtant, les études globales sur les polyosides solubles des moûts (Mourgues et Bénard, 1982 ; Brillouet, 1987) y ont rapporté la présence de mannose, de glucose et de xylose qui ne peuvent être attribués aux polyosides pectiques identifiés. Ces résidus pourraient provenir des hémicelluloses qui ont été identifiées dans les parois cellulaires du raisin (Saulnier et Thibault, 1987a ; Saulnier *et al.*, 1988 ; Lecas et Brillouet, 1994).

L'insolubilité de la cellulose et des hémicelluloses est une de leurs propriétés fondamentales et il faut recourir à des extractions alcalines dans des conditions drastiques pour les solubiliser, la cellulose restant généralement insoluble. On ne peut exclure toutefois la possibilité de la présence de fragments de mannanes, de xyloglucane ou d'arabinoxylanes dans les moûts et les vins. Le développement des travaux de recherche faisant appel aux techniques modernes d'isolement et de caractérisation rend probable l'identification prochaine de polyosides autres que pectiques dans le vin.

7.4.2 Polyosides produits par les micro-organismes

Mannoprotéines de levure

Les levures *Saccharomyces cerevisiae* ont un effet déterminant sur la composition polyosidique des vins. En effet, on observe une libération de mannoprotéines dans les moûts en fermentation, dès le début de la croissance des levures (Llaubères, 1987 ; Doco *et al.*, 1996b). Cette sécrétion de protéoglycannes n'est pas très bien comprise sur le plan de leur physiologie. Elle provoque un enrichissement important des vins en polymères contenant du mannose.

De nombreux auteurs se sont intéressés aux mannoprotéines retrouvées dans les vins. Il s'agit en fait d'une famille de molécules abondantes (100 à 150 $mg.l^{-1}$), assez homogène au point de vue de la composition qui est largement dominée par la présence de mannose, mais présentant une répartition de masses molaires extrêmement hétérogène (de 5 000 à plus de 400 000).

Si de nombreux travaux ont rapporté la présence des mannoprotéines, peu d'entre eux se sont attachés à détailler la structure fine des chaînes mannosidiques de fractions isolées à partir de vin. Ces études (Villetaz et Amadò, 1981 ; Waters *et al.*, 1994a) ont montré que l'organisation structurale des mannoprotéines présentes dans les vins correspond aux modèles largement décrits à partir des parois des levures (Ballou, 1982). Les mannoprotéines sont basées sur un noyau protéique qui représente en général moins de 20 % de la masse de la molécule, et qui porte deux types de chaînes glycanniques :

- de courtes chaînes linéaires de mannose lié en α-(1→3) ou α-(1→2) sont rattachées à la partie protéique au niveau de résidus de sérine ou de thréonine ;
- des chaînes polymannosidiques liées en α-(1→6), sont ramifiées par des chaînes latérales de mannose lié en α-(1→3) ou α-(1→2), et se rattachent à la partie peptidique par l'intermédiaire d'un N-Acétyl-chitobiose lié à un résidu d'asparagine.

Des techniques de libérations enzymatiques ou chimiques spécifiques permettent de différencier et quantifier ces deux types de chaînes (Waters *et al.*, 1994a).

Deux types de mannoprotéines existent dans les vins, qui peuvent être différenciés par leur origine ou leur mode d'obtention.

- Un premier type correspond aux mannoprotéines excrétées pendant la phase de croissance exponentielle des levures, qui s'accumulent durant la fermentation (Llauberes, 1987 ; Doco *et al.*, 1996b) pour se retrouver dans tous les vins à des concentrations proches de 100 $mg.l^{-1}$. Ce groupe de protéoglycannes a une très large répartition en masse molaire, une teneur moyenne en protéine proche de 20 % et une organisation structurale semblable à celles des mannoprotéines pariétales de la levure (Llauberes, 1987 ; Villetaz et Amadò, 1981 ; Waters *et al.*, 1994a). Elles peuvent être obtenues par culture de *S. cerevisiae* en milieu synthétique (Saulnier *et al.*, 1991).
- Un deuxième type comprend des mannoprotéines libérées lors de l'autolyse cellulaire des levures (cf. chapitre 11.1, sous chapitre autolyse), qui se produit lors du stockage du vin sur lies (Leroy *et al.*, 1990 ; Ledoux *et al.*, 1992). Elles peuvent être obtenues par extraction chimique (Lubbers *et al.*, 1993) ou enzymatique (Moine et Dubourdieu, 1996) à partir de parois isolées (écorces) de levure.

Les différences structurales existant entre ces deux types de constituants, dont les compositions globales sont proches, ne sont pas connues. Il semble pourtant que les mannoprotéines libérées par lyse cellulaire ou extraction à partir d'écorces de levure possèdent des propriétés protectrices, vis-à-vis des troubles protéiques et des précipités tartriques, que ne possèdent pas celles qui sont sécrétées par les levures en pleine croissance (cf. chapitre 10, sous chapitre protéases). Ces deux types de protéoglycannes levuriens doivent donc présenter des différences structurales fines non encore élucidées. Parmi les mannoprotéines relarguées par lyse cellulaire, une fraction de masse molaire de 30 000 semble plus particulièrement responsable des effets « colloïdes protecteurs » observés (Ledoux *et al.*, 1992 ; Moine et Dubourdieu, 1996).

De nombreuses propriétés sont attribuées à l'ensemble de ces constituants en œnologie qui seront décrites au paragraphe 7.6. Ces propriétés incluent des activités enzymatiques puisque l'invertase de levure est également une mannoprotéine (Lehle *et al.*, 1979).

Les parois de levure contiennent également des β-(1→3)-D-glucanes, leur libération dans le milieu est possible, bien qu'elle n'ait pas été observée directement. La présence de chitine semble par contre très peu probable en raison de la grande insolubilité de ce polymère linéaire de N-Acétyl-D-glucosamine. Ce monosaccharide n'est d'ailleurs pas observé dans les fractions polyosidiques des vins.

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

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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

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10.1021/jf9905020 CCC: $19.00 © 2000 American Chemical Society
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

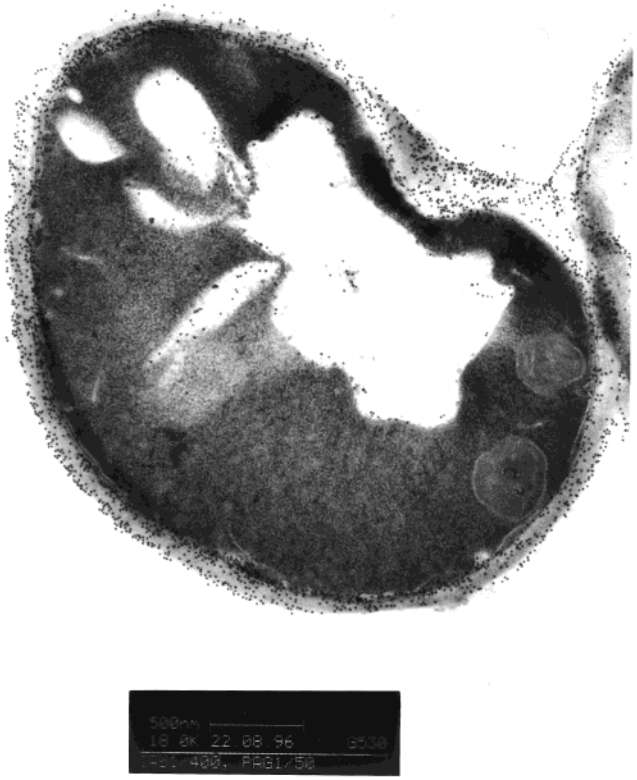

Figure 1. Site of HPF1 in *Saccharomyces cerevisiae* Maurivin PDM cells in late exponential phase**.** The binding of anti-HPF1 antibodies to the ultrathin sections of the cells were visualized as dense dots by binding of Protein A conjugated to colloidal gold to the yeast bound anti-HPF1 antibodies.

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).

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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

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 (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).

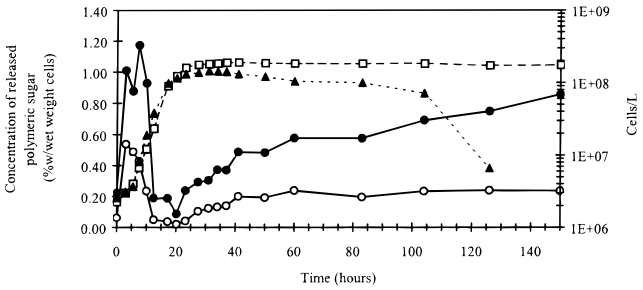

Figure 1. Change in the concentration of polymeric mannose (●) and polymeric glucose (○) in the culture supernatant during growth of PDM cells. Both the total cells (□) and viable cells (▲) are shown. The values are the mean of at least three analyses.

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.

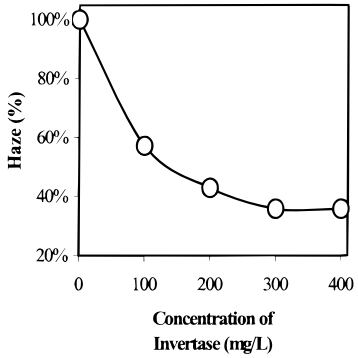

Figure 2. Effect of increasing addition of invertase on wine protein haze, measured as A_{490} and expressed as a percentage of the initial haze value (as observed with no invertase added). The values are the means of 4 analyses.

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 ($r^2 = 0.98$).

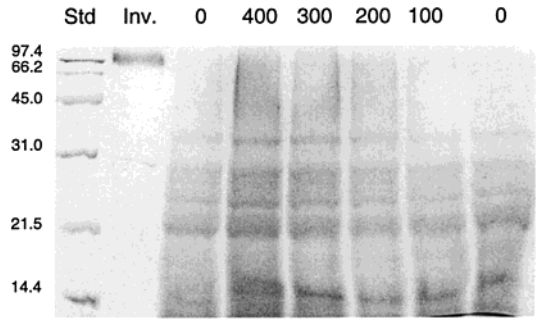

Figure 3. Protein composition as analyzed by SDS PAGE of hazes from 1 mL of wine with no added invertase (0) or with invertase added to the wine at 100−400 μg/mL (100−400). Invertase (100 μg, Inv.) and protein molecular weight standards (Std) were also analyzed. The M_r's of the protein molecular weight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

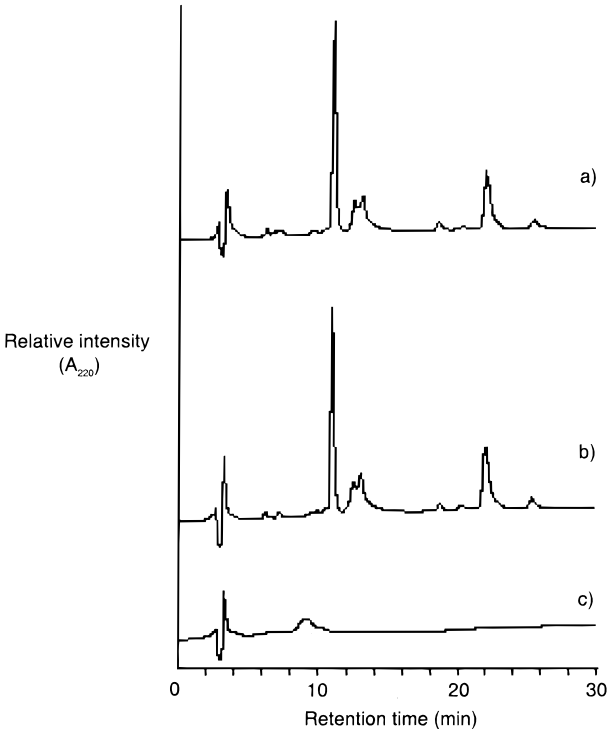

Figure 4. Protein composition of (a) unheated wine with no addition of invertase, (b) unheated wine with added invertase (400 mg/L), and (c) heated wine with added invertase (400 mg/L) as assessed by HPLC analysis.

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

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Management des mannoprotéines

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Management of mannoproteins

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Mannoproteinmanagement

Résumé

Les mannoprotéines de levures issues de la paroi cellulaire sont libérées au cours de la fermentation alcoolique et de l'élevage sur lies. Elles contribuent à la stabilité microbiologique et physico-chimique des vins. Différentes techniques de production industrielle de ces mannoprotéines ont été mises au point. L'autorisation de leur utilisation est actuellement en cours d'étude à l'O.I.V.

Summary

Mannoproteins issuing from the yeast cell wall are released during alcoholic fermentation and ageing of wines in contact with yeasts. They contribute to microbial and physico-chemical stability of the wines. Various industrial techniques for the production of mannoproteins have been developed. Their use in winemaking is is being evaluated by the O.I.V.

Zusammenfassung

Die aus den Zellwänden der Hefen stammenden Mannoproteine werden während der alkoholischen Gärung und dem Ausbau des Weines auf der Hefe freigesetzt. Sie tragen zur mikrobiologischen und physiko-chemischen Stabilität des Weines bei. Es wurden verschiedene Techniken zur industriellen Gewinnung solcher Mannoproteine entwickelt. Die Zulassung von Mannoproteinen für die Weinbereitung wird zur Zeit vom O.I.V. geprüft.

I. Les mannoprotéines de la paroi de *Saccharomyces cerevisiae*

La paroi de *Saccharomyces cerevisiae* contient trois constituants: glucane, chitine et mannoprotéines. Le β1,3-glucane (Smits et al,1999) constitue le squelette de la paroi sur lequel sont attachées par liaisons covalentes deux types de mannoprotéines. Les premières liées directement au β1,3-glucane appartiennent à la famille des PIR-protéines (protéines avec des répétitions internes), les secondes qui lui sont associées par l'intermédiaire de β1,6-glucane appartiennent à la famille des GPI-protéines (protéines à ancre glycérophosphate inositol). Une troisième famille de mannoprotéines, correspond à celles qui peuvent être extraites des parois soit par le dodécylsulfate de sodium ou un agent réducteur. Elles sont liées par ponts disulfure aux autres protéines de la paroi ou par liaisons non covalentes aux autres composants de la paroi (Fig.1).
Les mannoprotéines sont en fait des glycopeptides contenant en moyenne 80% d'oses représentés essentiellement par du mannose, un peu de glucose et 20% d'acides aminés.

II. Libération des mannoprotéines au cours de la vinification

Les mannoprotéines sont libérées d'une part au cours de la fermentation alcoolique d'autre part au cours de la conservation des vins sur lies.
Au cours de la fermentation alcoolique (Fig. 2), la multiplication des levures par bourgeonnement nécessite une hydrolyse partielle des parois cellulaires sous l'action de glucanases qui entraîne la libération de mannoprotéines. Cette production de mannoprotéines dépend de différents facteurs.
De nombreux auteurs (Llaubères,1987,Rosi et al, 1998) ont montré qu'elle variait en fonction de la souche de *Saccharomyces cerevisiae*, ce que avons vérifié au cours de la fermentation d'un moût synthétique. (Tab.1), certaines souches sont capables de produire deux fois plus de mannoprotéines que d'autres.
Nous avons également montré (Boivin et al, 1998) que la libération de mannoprotéines par *Saccharomyces cerevisiae* dépendait du degré de clarification des moûts (Fig. 2). Plus un moût est clarifié plus les levures produisent de mannoprotéines (Tab. 2).
Lors de la conservation des vins sur lies telle qu'elle est pratiquée au cours de l'élevage des grands vins blancs de Bourgogne avec remise en suspension des lies par batonnage et lors de l'élaboration des vins effervescents par la méthode traditionnelle intervient le phénomène d'autolyse. Cette autolyse correspond à une destruction de la cellule par ses propres enzymes et notamment à une hydrolyse de la paroi sous

l'action des glucanases (Charpentier et al 1986, Charpentier et Freyssinet 1989) avec libération de mannoprotéines associées à des polymères de glucose (Tab 3, 4).

III. Propriétés œnologiques des mannoprotéines

Les mannoprotéines levuriennes interviennent sur la stabilité microbienne et physicochimique des vins notamment sur le déroulement de la fermentation malolactique, la stabilité des protéines, du bitartrate de potassium, ainsi que sur les combinaisons des arômes et des composés phénoliques.

Activation de la fermentation malolactique

Lors des vinifications en blanc suivies en Bourgogne, nous avions constaté que des fermentations alcooliques longues souvent liées à une mortalité importante des levures étaient suivies de fermentations malolactiques plus rapides. Ces observations nous ont amenés à enrichir un milieu de culture pour bactéries lactiques avec des mannoprotéines de levures. Il est apparu (Fig. 3) que l'ajout de mannoprotéines de levures permet d'obtenir une population bactérienne accrue. Les mannoprotéines sont en effet dégradées par *Oenococcus oeni* à qui elles servent de source de sucre et d'azote. (Guilloux-Bénatier et al 1995).

Amélioration de la stabilité protéique

Deux équipes ont mis en évidence le rôle stabilisateur des mannoprotéines vis à vis des troubles liés aux protéines. Waters et al (1993, 1994), Moine-Ledoux (1996) ont en effet montré que le trouble obtenu par chauffage des vins blancs diminue en présence de mannoprotéines. (Fig. 4) et que l'ajout de mannoprotéines permet de réduire les doses de bentonite nécessaires pour obtenir la stabilité protéique (Fig. 5)

Amélioration de la stabilité tartrique

La stabilité tartrique d'un vin peut être évaluée par différentes techniques et notamment par mesure de conductivité (Maujean et Vallée 1985). Cette technique nous a permis (Lubbers et al.,1993, Feuillat et Charpentier,1998) de déterminer l'influence de différentes mannoprotéines commerciales sur la température de cristallisation des sels de potassium et de montrer qu'elles la diminuent (Tab. 5)

Association des mannoprotéines avec les composés d'arôme

Lubbers et al (1994) ont mis en évidence l'existence d'interactions entre mannoprotéines libérées au cours de la fermentation alcoolique et composés d'arômes par la technique de dialyse équilibre. (Fig. 6) Seules les mannoprotéines dont la fraction

protéique est plus importante (fraction NF) fixent la β-ionone et une quantité plus élevée d'hexanoate d'éthyle. Lavigne et Dubourdieu (1996) ont étudié la fixation des composés soufrés (méthanethiol et éthanethiol) par des parois de levures dépourvues ou non de mannoprotéines (Fig.7) et ont confirmé ainsi le rôle des mannoprotéines dans la fixation de ces composés.

Combinaison des mannoprotéines avec les composés phénoliques

Escot et al (2001) ont étudié l'influence des mannoprotéines produites par les levures sur les propriétés des polyphénols. Un vin rouge de cépage Pinot noir a été additionné de différentes quantités de mannoprotéines produites par trois souches de *Saccharomyces cerevisiae* au cours de la fermentation alcoolique et de l'autolyse. Seuls les résultats obtenus avec un ajout de 200mg/l sont présentés dans le tableau 6. On constate que ces additions entraînent une diminution de l'indice de gélatine, notamment pour les mannoprotéines issues de la souche BM 45. Cette diminution due à la combinaison entre tannins et mannoprotéines, se traduit par une diminution de leur astringence. L'ajout de mannoprotéines provoque une augmentation de l'indice d'éthanol due une augmentation de la combinaison entre la fraction polysaccharidique des mannoprotéines et les tannins. Les variations de l'indice de PVPP (polyvinylpolypyrrolidone) ne sont pas significatives. Ces résultats ont été confirmés par évaluation sensorielle des vins, les vins additionnés de mannoprotéines ont été décrits comme moins astringents.

IV. Préparation et production de mannoprotéines industrielles

Les mannoprotéines de levures sont produites industriellement par différentes techniques protégées par plusieurs brevets nationaux et internationaux. Ces techniques font intervenir soit un traitement par la chaleur dans un tampon à pH7 soit une extraction enzymatique par des glucanases, ces deux traitements étant appliqués à des levures entières ou à des écorces (Tab. 7).

Une étude de la composition de ces mannoprotéines et de leur effet stabilisateur vis à vis des précipitations tartriques a été effectuée par Feuillat et Charpentier (1998). Il apparaît que ces mannoprotéines préparées suivant les données de la littérature diffèrent par l'importance de leurs fractions protéiques (Tab. 8).

Les groupes d'experts «Technologie» et «Microbiologie» de l'O.IV. ont donné une recommandation favorable concernant l'utilisation des mannoprotéines de levures pour la stabilisation tartrique des vins blancs. Actuellement une monographie concernant ces mannoprotéines d'addition est en cours d'étude en vue de sa parution dans le code œnologique international.

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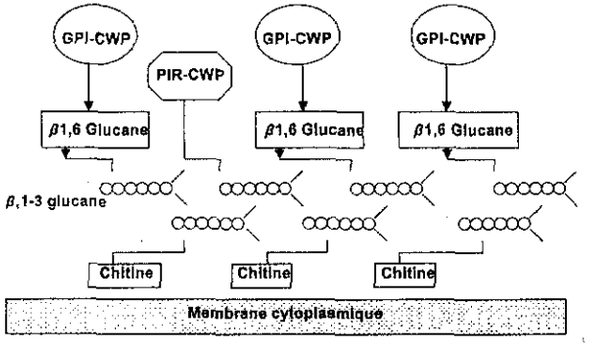

Figure 1: Schéma de la paroi de Saccharomyces cerevisiae (d'après Smith G.J., Kapteyn J.C., Van den Ende and Klis F. 1999)

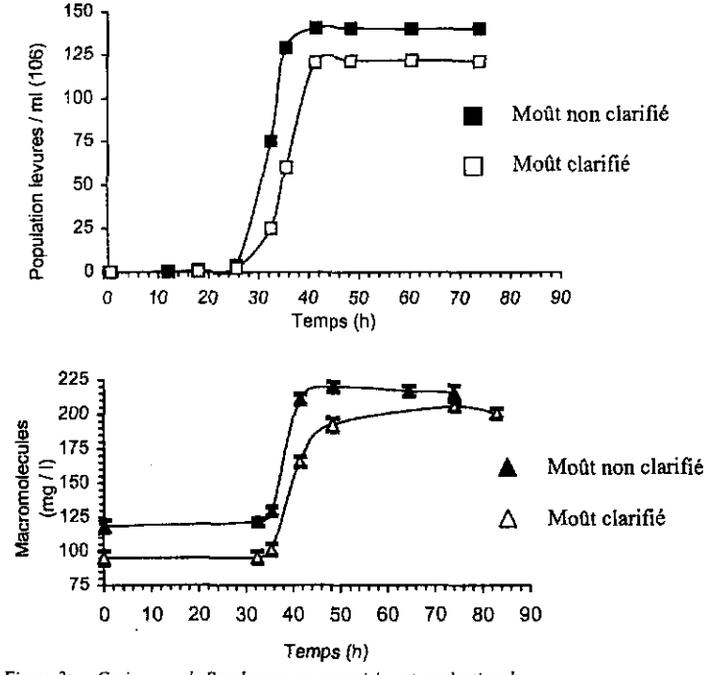

Figure 2: Croissance de Saccharomyces cerevisiae et production de mannoprotéines dans des moûts clarifié et non clarifié

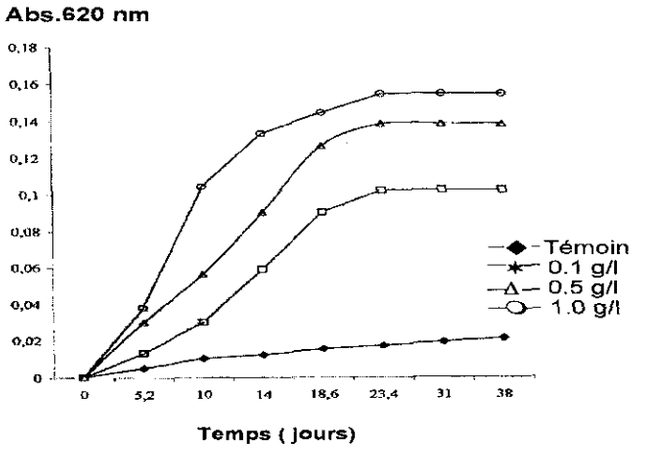

Figure 3: Influence de l'ajout de mannoprotéines sur la croissance d'Oenococcus oeni (D'après Guilloux-Bénatier et al.)

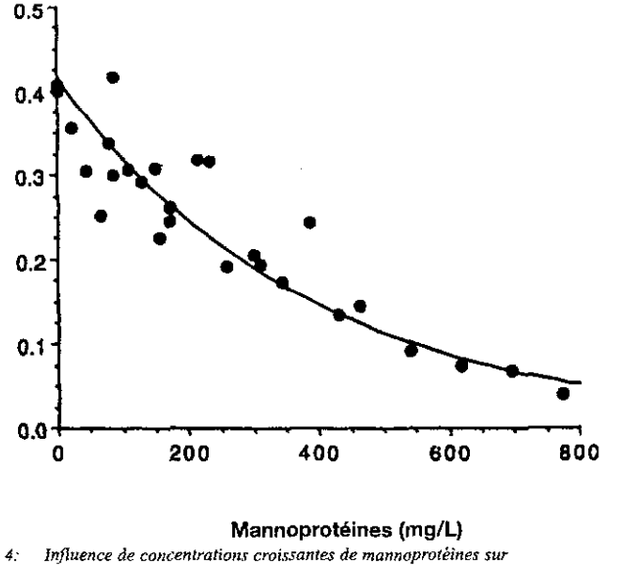

Figure 4: Influence de concentrations croissantes de mannoprotéines sur l'intensité du trouble protéique obtenu après chauffage du vin (D'après Waters et al.)

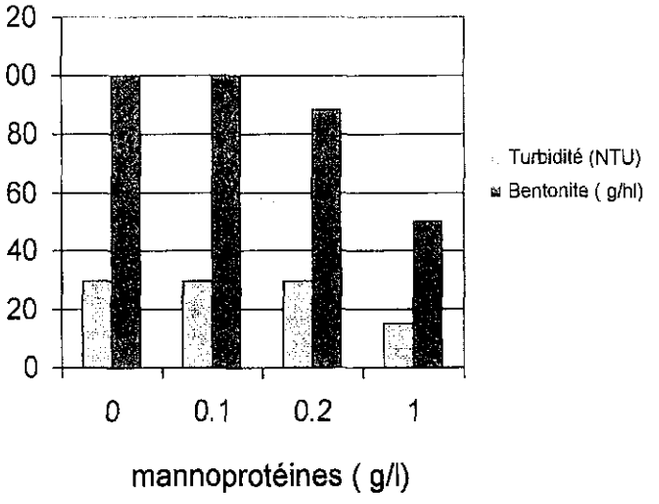

Figure 5: Influence de la teneur en mannoprotéines sur les quantités de bentonite à ajouter pour obtenir la stabilité protéique (D'après Moine-Ledoux)

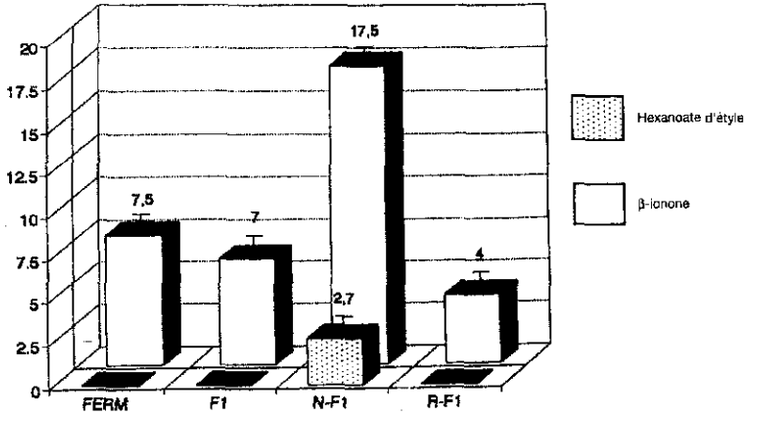

Figure 6: Fixation d'arômes par les mannoprotéines libérées par les levures au cours de la fermentation alcoolique

Ferm totalité des macromolécules libérées au cours de la fermentation alcoolique

F1 fraction de Ferm obtenue par chromatographie d'échange d'ions

N-F et R-F fractions obtenues après séparation des macromolécules par chromatographie d'affinité sur Concanavaline A.

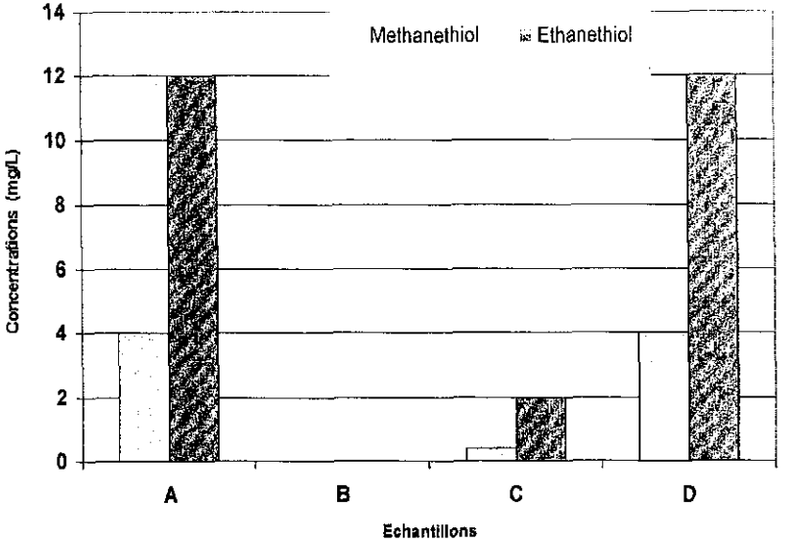

Figure 7: Influence des mannoprotéines de la paroi de levures sur la fixation de composés soufrés (Lavigne et al 1996)

A - Tampon + méthanethiol + éthanethiol

B - Tampon + méthanethiol + éthanethiol + lies

C - Tampon + méthanethiol + éthanethiol + parois de levures

D - Tampon + methanthiol + éthanethiol + parois de levures dépouvues de mannoprotéines

Saccharomyces cerevisiae	Concentration en sucres (g/L)	Mannoprotéines produites (mg/100mg biomasse sèche)
BRG	170,0	0
	70,1	1,51
	0,7	3,10
RC 212	170,0	0
	66,0	0,02
	2,1	1,32
BM 45	170,0	0
	66,5	1,50
	1,2	3,54

Tableau 1: Influence de la souche de levure sur la production de mannoprotéines au cours de la fermentation alcoolique

	Moût non clarifié	Moût clarifié
Turbidité initiale (NTU)	380	34
Population levures 106/ml	140 ± 3	120 ± 2
Viabilité en fin de fermentation (%)	89 ±2	74 ± 1
Production de mannoprotéines (µg/106 levures)	0,67	0,87

Tableau 2: Production de mannoprotéines au cours de la fermentation alcoolique, influence de la turbidité du moût

	Colloïdes totaux (mg/L)
Vin conservé sur lies fines	809
Vin conservé sur lies totales	893
Vin conservé sur lies totales avec batonnage	930

Tableau 3: Influence de la conservation sur lies et du batonnage sur la teneur en colloïdes d'un vin de Meursault conservé en fût pendant 8 mois

	Mannoprotéines (mg/L)
Vin de base	366
Vin après tirage	500
Conservation sur levures	
1 mois	408
2 mois	493
3 mois	550
4 mois	567
5 mois	580
6 mois	602

Tableau 4: Influence de la conservation sur levures sur la teneur en mannoprotéines des vins effervescents

	Température de cristallisation (°C)	Δt °C
Vin blanc sans addition	8,20	
+ mannoprotéine A	6 ,60	-1,60
+ mannoprotéine D	6,90	-1,30
+ mannoprotéine FS	6,00	-2,20
+ mannoprotéine MP1	7,20	-1,00
+ mannoprotéine MP2	7,10	-1,10
+ mannoprotéine GB	5,85	-2,35

Tableau 5: Influence de l'ajout de différentes mannoprotéines(250 mg/L) sur la température de cristallisation du bitartrate de potassium et du tartrate de calcium (D'après Feuillat et Charpentier)

	Saccharo myces	Indice de gélatine (%)	Indice de PVPP(%)	Indice d'éthanol(%)
Vin témoin sans addition		68,2	32,7	6,0
Vin + 200mg/l de mannoprotéines (fermentation)	BRG	23,9	44,0	10,5
	RC 212	58,2	37,2	5,7
	BM 45	21,7	57,0	12,8
Vin + 200mg/l de mannoprotéines (autolyse)	BRG	29,0	33,0	10,5
	RC 212	27,5	36,2	7,2
	BM 45	22,8	34,0	10,3

Tableau 6: Influence des mannoprotéines produites par différentes souches de Saccharomyces sur les propriétés des polyphénols

Origine	Extraction		Mannoprotéines
Levures entières	Chauffage 125°C dans tampon pH 7, lyophilisation	Herlin	A
	Chauffage 125°C dans tampon pH 7, précipitation à l'éthanol	Herlin	D
	Glucanex, lyophilisation	Moine-Ledoux	FS
Ecorces de levures	Chauffage dans l'eau, 3 heure à 100 °C, lyophilisation	Gerbaud	MP1
	Chauffage dans l'eau, 3 heure à 100 °C, précipitation à l'éthanol	Gerbaud	MP2
Inconnue	Physico-chimique	Gist-Brocades	GB

Tableau 7: Techniques de préparation des mannoprotéines décrites dans la littérature

Mannoprotéines	Oses (glucose)	Protéines (BSA)
A	70,3	28,6
D	60,1	12,5
FS	87,5	7,0
MP1	73,6	14,0
MP2	86,0	6,6
GB	84,8	15,3

(tous les résultats sont exprimés en % du poids sec)

Tableau 8: Composition des différentes mannoprotéines extraites de levures entières ou d'écorces de levures par chauffage ou traitement enzymatique.

Notices

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Notizen

Association Internationale d'Oenologie,de
Gestion d'Entreprise et de Marketing du Vin

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International Association of Enology,
Management and Wine Marketing

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Internationale Vereinigung für Oenologie,
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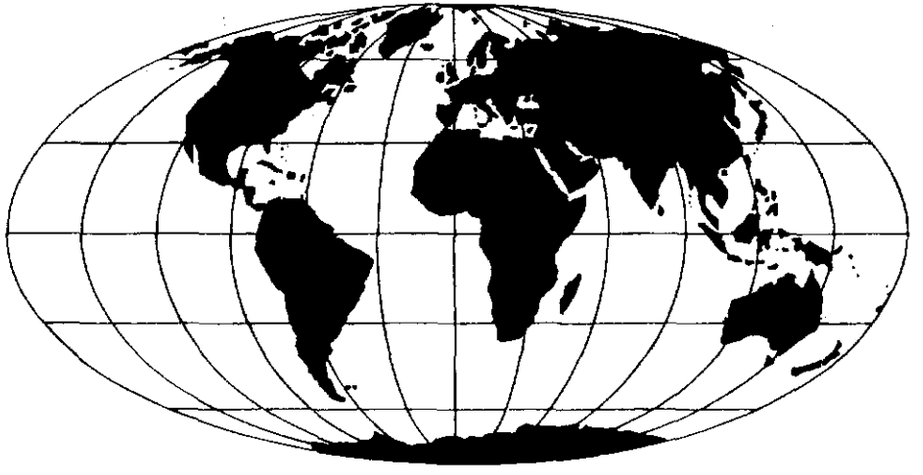

13ème Symposium International d'Oenologie
09 – 12 Juin 2002

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13th International Enology Symposium
June 09th – June 12th 2002

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13. Internationales Oenologisches Symposium
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École Nationale Supérieure d'Agronomie (ENSA)
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