JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of White Wine Mannoproteins

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Mannoproteins, *Saccharomyces cerevisiae* yeast polysaccharides, play a major role in wine processing and characteristics. A systematic characterization of these polymers in terms of chemical composition and molecular structure is addressed in this study. Mannoproteins were isolated from white wine through a sequence of operations that consisted of nanofiltration for concentration of macromolecules, polysaccharide precipitation with ethanol, and affinity chromatography on concanavalin A. The whole wine mannoproteins present a very broad molecular mass distribution with several populations. Two major populations with very different compositions were separated by size exclusion chromatography. The mannoproteins with higher molecular mass are a mannose homopolymer containing 10.3% protein. The mannoproteins with lower molecular mass consisted of 87.5% of mannose and some other residues and a protein content of 2.5%. The highest molecular weight mannoprotein structure was examined by ^{1}H and ^{13}C NMR spectroscopic techniques such as 1-D TOCSY, 2-D COSY, and 2-D HMQC.

KEYWORDS: White wine; mannan; mannoprotein; NMR spectroscopy

INTRODUCTION

Mannoproteins are polysaccharides produced by *Saccharomyces cerevisiae* yeast during alcoholic fermentation. These polymers are present in significant amounts in the wines, and their concentration depends on the wine-making process (*1*). Mannoproteins play a very important role in wine characteristics and processing, namely, in the operations of membrane filtration and tartaric stabilization. Adsorption experiments with polymeric membranes proved that mannoproteins have a significant fouling effect (*2*). Moreover, studies with model solutions showed that the high molecular mass mannoproteins induce severe reductions of permeation fluxes during membrane filtration (*3*). On the other hand, the eventual removal of mannoproteins in wine filtration can affect wine organoleptic properties and tartaric stability. In fact, mannoproteins act as natural inhibitors of potassium hydrogen tartrate crystallization, preventing the occurrence of precipitates in wine (*4*−*7*). Mannoproteins prevent the formation of protein haze in the wines (*8*−*10*) and also seem to have a role in the volatility of aroma compounds (*11*, *12*). To understand the role played by wine mannoproteins in wine processing, a systematic characterization of these polymers in terms of chemical composition and molecular structure is necessary and constitutes our object of concern.

Most of the data present in the literature refer to the characterization of the mannoproteins found in *Saccharomyces cerevisiae* yeast cell walls (*13*−*15*). There has been some research on the characterization of exocellular mannoproteins released during the fermentation in synthetic media that simulated must (*14*, *16*). These studies show that the mannoproteins bound on concanavalin A consisted mainly of mannose and small amounts of glucose, associated with 10−20% of protein. There are very few studies of the isolation and characterization of wine mannoproteins. Pellerin et al. (*17*) fractionated the wine polysaccharides by charge and isolated the mannoproteins corresponding to those fractions by means of concanavalin A. They found that these polymers were almost pure mannans (mannose content >95% of the total sugar content) and represented several populations over a wide range of molecular weights. Waters et al. (*8*) isolated and characterized a high molecular weight mannoprotein of 420 kDa, which was very rich in mannose (98%) with 30% protein content. These authors found by methylation analysis that this mannoprotein presented a very branched structure as most of the mannoses were terminal and the most abundant linkage types wereq $1 \rightarrow 2$, $1 \rightarrow 3$, and $1 \rightarrow 2,6$.

In previous work (*8*, *17*), the wine polysaccharides were fractionated by charge prior to the isolation of mannoproteins by means of concanavalin A. In the present work, wine macromolecules were first concentrated by nanofiltration and then the polysaccharides were purified by successive ethanol precipitations. The wine mannoproteins were isolated directly from the whole wine polysaccharides by affinity chromatography on concanavalin A. This approach allows the isolation of mannoproteins with the same molecular mass distribution as

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10.1021/jf0202741 CCC: $22.00
Published on Web 09/12/2002

they have in the wine. The molecular mass distribution of the whole wine mannoproteins was assessed by size exclusion chromatography (SEC) with multiangle laser light scattering (LS) and refractive index (RI) detectors. To characterize the different molecular weight wine mannoproteins, they were further fractionated by semipreparative size exclusion chromatography. The fractions obtained were characterized in terms of sugar residues and protein content. The investigation of the structure of these polymers was carried out by NMR techniques.

MATERIALS AND METHODS

Separation and Purification of Polysaccharides. Mannoproteins were isolated from a white wine, Vinho Verde, obtained from Loureiro grapes harvested in 1999. The juice was fermented using the yeast strain QA23 (Lallemand, Rexdale, Canada) at a constant temperature of 16 °C. A volume of 8 L of wine was concentrated using a Ropur TS60 (Ropur AG, Münchenstein, Switzerland) nanofiltration membrane with a molecular weight cutoff ~1 kDa. The wine concentration was performed in a Dow laboratory unit M20 (DSS, Nakskov, Denmark) with plate and frame membrane arrangement with 0.072 m^2 of membrane surface area. Temperature was set at 25 °C and transmembrane pressure at 10 bar. This operation was run in concentration mode, that is, the concentrate was recirculated to the feed tank while permeate was continuously drained out. This operation mode and the membrane molecular weight cutoff allowed the concentration of macromolecules and the removal of ethanol, salts, and small molecules. Salt removal was controlled by the measurement of the conductivity in the concentrate compartment. During the concentration operation, the permeate flux declines. When the permeation flux dropped below 8 L/h/m^2, 2 L of water were added to the concentrate to purify the macromolecules. No further water was added when the concentrate conductivity was lower than 1000 μS/cm, and wine was concentrated to a final volume of 500 mL.

The concentrate of wine macromolecules was subjected to a purification procedure as follows: Precipitation of the polysaccharides was achieved by the addition of ethanol. A volume of 1000 mL of ethanol was added to the concentrate of wine macromolecules and the suspension kept for 24 h at 4 °C. The suspension was then centrifuged at 150*g*. After centrifugation, the supernatant was discarded and the precipitate washed two times with ethanol; the precipitate was vacuum-dried at room temperature during 48 h. The product obtained had some coloration and was then subjected to further precipitation. The solid was dissolved in water, and a new sequence of precipitation, centrifugation, and drying was performed as previously described. A slightly colored powder of wine polysaccharides was obtained and used for the subsequent isolation of the mannoproteins.

Isolation of Mannoproteins. The mannoproteins were specifically adsorbed on a 50 × 16 mm column of concanavalin A Sepharose (Pharmacia, Uppsala, Sweden). The polysaccharides were dissolved in water and centrifuged, and the supernatant was loaded onto the column using a 0.05 M acetate buffer of pH 5.6 containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 0.15 M NaCl. The elution was monitored using a Gilson model 133 (Gilson, Villier le Bel, France) refractive index detector. After the signal had returned to zero (complete elution of the non-adsorbed polysaccharides), the mannoproteins were recovered by eluting with the eluent described above containing 0.5 M methyl α-D-mannopyranoside (Sigma, St. Louis, MO). The eluent containing the mannoproteins was dialyzed against water. The sample was concentrated under vacuum and freeze-dried.

Analytical Methods. The saccharide residues were quantified by GC analysis of alditol acetates as described by Albersheim (*18*), using inositol as internal standard. The alditol acetates were separated and quantified on a Hewlett-Packard 9580 gas chromatograph with a 30 m × 0.53 mm Supelco SP-2380 column (Sigma-Aldrich, St. Louis, MO). The column temperature was initially set at 195 °C and raised to 225 °C at 2.5 °C/min. Nitrogen was used as carrier gas at 4 mL/min. The total protein content was determined as described by Lowry (*19*), and the quantification was achieved through calibration with bovine serum albumin fraction V from Merck (Darmstadt, Germany).

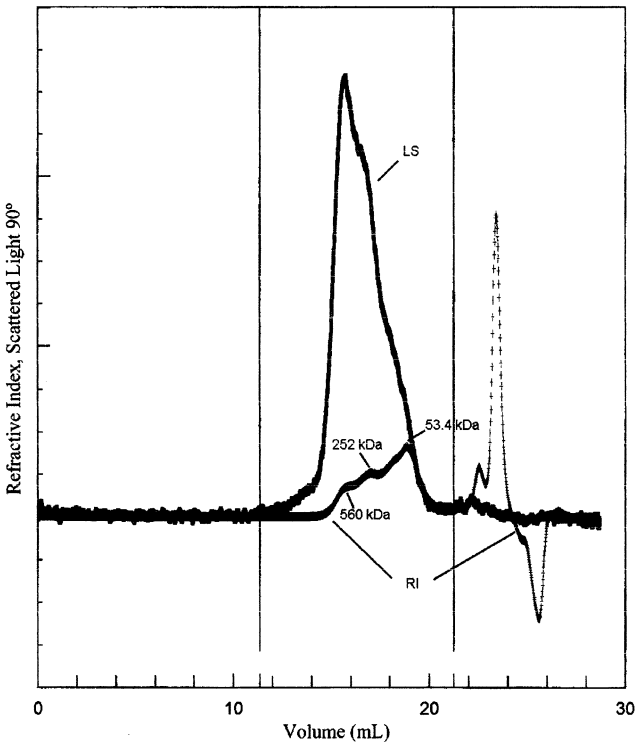

Figure 1. Molecular mass determination of wine mannoproteins by size exclusion chromatography. RI, refractive index; LS, scattered light at 90°; columns, Shodex OHpak B-804 and B-805; eluent, 0.1 M $NaNO_3$; flow rate, 0.5 mL/min; injection volume, 100 μL; concentration, 2 mg/mL; *T*, 30 °C.

High-Performance Size Exclusion Chromatography (HPSEC). The molecular weight distribution of wine mannoproteins was determined by HPSEC on a 150C Waters apparatus fitted with a differential refractometer (Waters, Milford, MA) and a DAWN DSP-F multiangle laser light scattering detector (Wyatt, Santa Barbara, CA), on-line. The eluent was 0.1 M $NaNO_3$ solution. Shodex OHpak B0 804 and B0805 300 × 7.5 mm columns (Showa Denko, Tokyo, Japan) connected in series were used. The preparative fractionation of the mannoproteins was performed with a set of two columns in series: a 600 × 7.5 mm TSK G3000 PW column (Tosoh, Tokyo, Japan) and a 500 × 7.5 mm Shodex OHpak B-805 (Showa Denko). The elution was monitored with a Waters 2410 refractive index detector.

NMR Spectroscopy. The samples were dissolved in D_2O. 1-D ^1H and ^{13}C spectra were recorded at 80 °C with an AC 300 Bruker instrument (300 MHz) equipped with a 5 mm dual probe. The 1-D total correlation spectroscopy (TOCSY), gradient correlated spectroscopy (g-COSY), and 2-D $^{13}C-^1H$ heteronuclear multiple-quantum correlation (HMQC) experiments were carried out with an Avance 400 Bruker (400 MHz) equipped with a 5 mm BBIZ probe, using the standard pulse sequences. ^1H NMR chemical shifts are expressed in parts per million (δ) by reference to internal acetone ($\delta_H = 2.225$ ppm), and ^{13}C NMR chemical shifts are given relative to external tetramethylsilane, TMS ($\delta_C = 0$ ppm).

RESULTS AND DISCUSSION

The sequence of concentration of the wine macromolecules and ethanol precipitation of the polysaccharides yielded 1.551 g of polysaccharides from a batch of 8 L of wine. This corresponds to a wine polysaccharide content of 0.194 g/L. The isolation of mannoproteins with concanavalin A yielded 0.3931 g of mannoproteins from a sample of 1.2178 g of wine polysaccharides. The mannoproteins represent 32.2% of the total wine polysaccharides.

The size exclusion chromatogram of the whole wine mannoproteins is displayed in **Figure 1**. Three groups of manno-

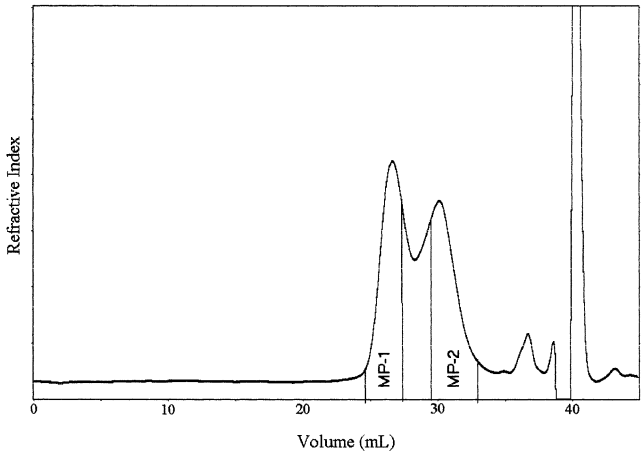

Figure 2. Preparative fractionation of wine mannoproteins (MP) by SEC (columns, TSK G3000 PW and Shodex OHpak B-805; eluent, 0.1 M $NaNO_3$; flow rate, 1 mL/min; injection volume, 250 μL; concentration, 10 mg/mL; *T*, 30 °C.

proteins were identified, with molecular weights of 53.4, 252, and 560 kDa, respectively. These wine mannoproteins covering different ranges of molecular weights are probably associated with different chemical compositions.

To investigate the chemical composition of the different molecular weight mannoproteins, the whole wine mannoproteins were fractionated by semipreparative size exclusion chromatography. The column set chosen allowed the fractionation of the wine mannoproteins in two peaks, MP-1 and MP-2, as shown in **Figure 2**. As the two peaks were still partially overlapped, the intersection region was discarded and the two polymers were recovered separately. **Table 1** displays the chemical composition in terms of sugar residues and protein content for the two fractions, MP-1 and MP-2, and for the sample of mannoproteins before fractionation, MP. This all-wine mannoprotein sample had a high mannose content of 87.6% and contained other sugar residues consisting of rhamnose, arabinose, galactose, and glucose. The fraction of mannoproteins with the highest molecular weight, MP-1, was a mannose homopolymer, that is, a pure mannan. The one with the lowest molecular weight, MP-2, had a high content of mannose, and other sugars were also present. Besides the differences in sugar residues composition, the protein contents were also very different between the two fractions. Most of the protein present in the mannoproteins is associated with the highest molecular weight macromolecules. These results are in agreement with the findings of Waters et al. (*8*), who isolated a high molecular mass mannoprotein from red wine mainly composed of mannose with high protein content.

Table 1. Composition of Wine Mannoproteins[a]

	sugar residues[b]					
	rhamnose	arabinose	mannose	galactose	glucose	protein[c]
MP	1.6	4.1	87.6	5.3	1.4	6.2
MP-1			100			10.3
MP-2	1.9	2.9	87.5	5.0	2.6	2.5

[a] MP, total wine mannoproteins; MP-1 and MP-2, high and low molecular weight mannoprotein fractions, respectively. [b] Percentage of sugars in the polysaccharide. [c] Percent of dry matter.

The occurrence of glucose was expected, as it has also already been reported for the mannoproteins released by the yeast in synthetic media, namely, in the low molecular weight macromolecules fraction (*14*). However, one would not expect the presence of other sugar residues besides mannose and glucose in the wine mannoproteins. In fact, as displayed in **Table 1** the MP-2 fraction contains small percentages of other sugars: rhamnose, arabinose, and galactose. These sugars are from the other wine polysaccharides, namely, arabinogalactanproteins (AGP), molecular weight ~150 kDa, and rhamnogalacturonans type II (RGII), molecular weight ~10 kDa. Despite the high affinity of the concanavalin A for the mannoproteins, these polysaccharides were also partially adsorbed by the lectin. They are present in the MP-2 fraction due to their relatively low

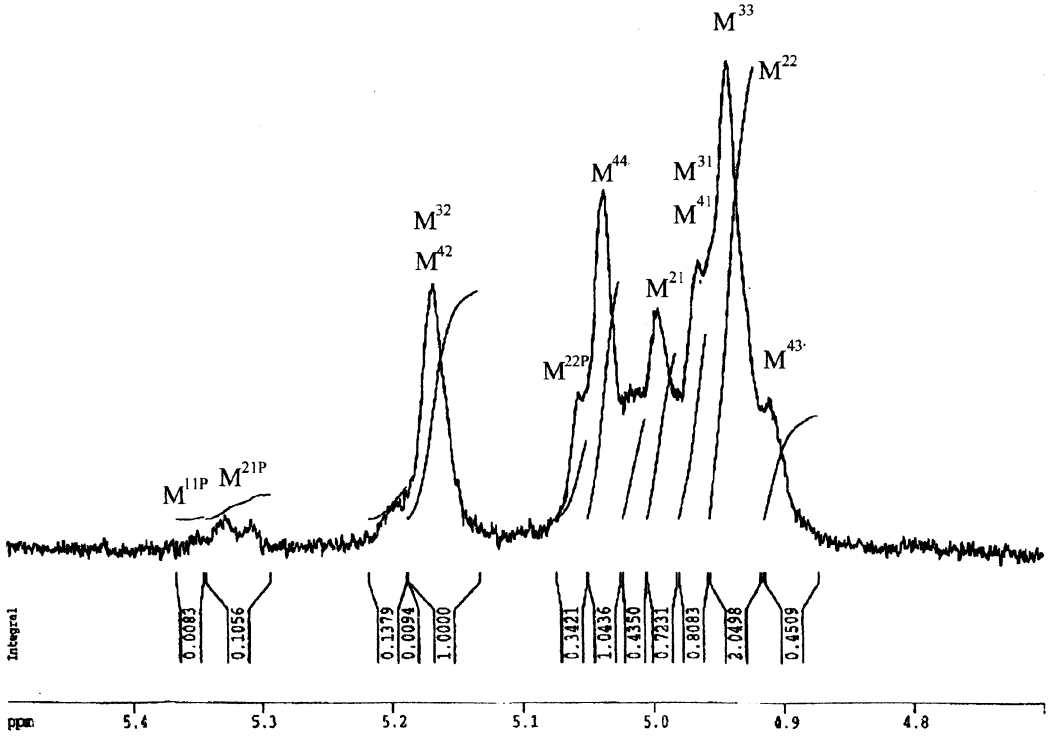

Figure 3. Anomeric resonance in the ^{1}H NMR spectrum of MP-1.

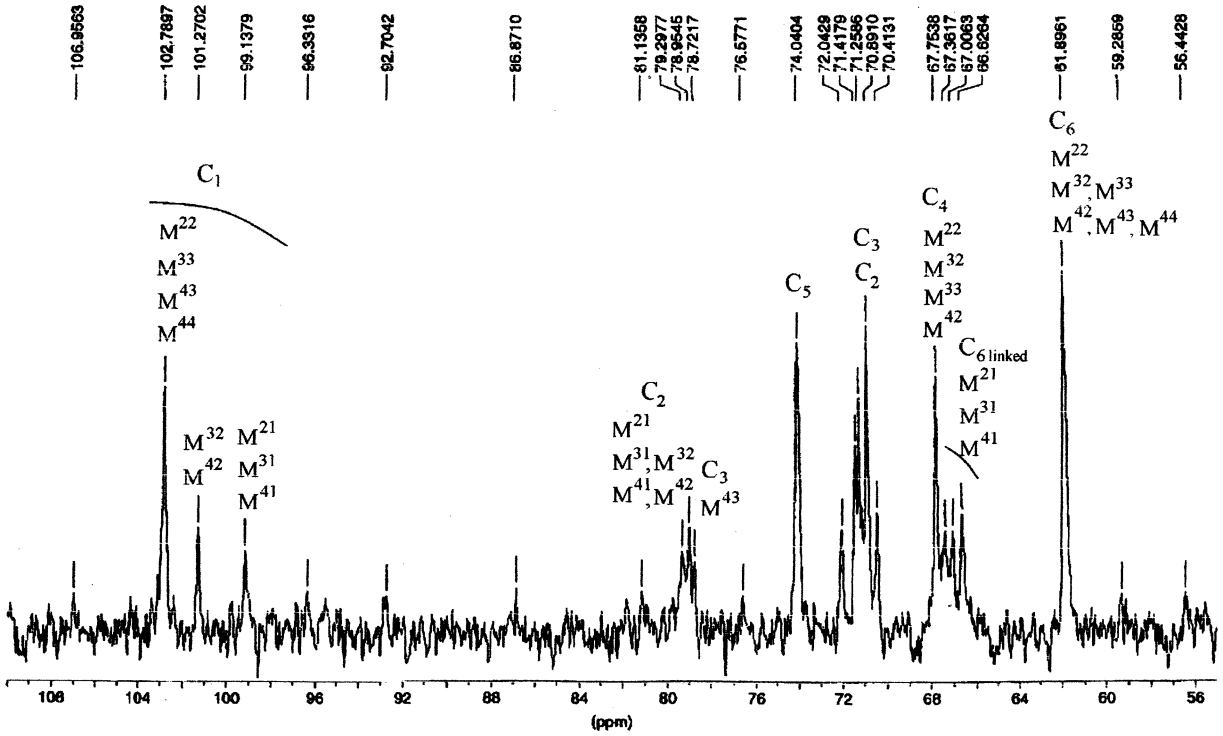

Figure 4. ^{13}C NMR spectrum of the MP-1 fraction.

molecular weights. More evidence for the presence of these polysaccharides comes from the ^{1}H NMR spectrum of MP-2, which shows peaks corresponding to several sugar residues with different linkages, indicating therefore the presence of a mixture of polymers. Further purification of MP-2 is needed to determine the low molar mass mannoproteins structure.

As the MP-1 fraction corresponded to a pure mannan, its structure was investigated. The MP-1 ^{1}H and ^{13}C NMR spectra are presented in **Figures 3** and **4**. As there are no data in the literature on NMR spectra of mannoproteins extracted from wine, MP-1 NMR spectra were compared with data for the mannan obtained directly from *S. cerevisiae* yeast. The NMR data of intact *S. cerevisiae* mannan present in the literature allowed the assignment of the MP-1 NMR spectra, on the basis of the structure proposed by by Vinogradov et al. (*15*). The assignment of the anomeric region of ^{1}H NMR spectrum of MP-1 is shown in **Figure 3**. Mannose residues are represented by the letter M followed by two digits. The first digit indicates the length of the side chain including the mannose residue in the backbone, and the second digit refers to the position of the sugar, 1 being the backbone sugar residue. The letter P indicates a phosphorylated mannose. An attempt at complete assignment of the ^{1}H spectrum was undertaken with 1-D TOCSY and g-COSY experiments. Due to the complexity of this structure, only H-2 and H-3 signals were located (**Table 2**), in agreement with results previously published by Vinogradov et al. (*15*). In the same way, the ^{13}C spectrum is attributed as shown in **Figure 4**. Chemical shift assignments of different C-2 and C-3 atoms are corroborated by results of 2-D $^{13}C-^{1}H$ HMQC experiments (**Table 2**). It was not possible to identify the ^{1}H signal of GlcN because it was hidden by the water signal. The ^{31}P spectrum was also recorded. Due to the poor resolution, quantification of phosphoesters was impossible.

The NMR results show a very good agreement with the data reported by Vinogradov et al. (*15*), meaning that the high molecular weight mannoproteins present in wine have a molecular structure very similar to the intact yeast mannans.

Table 2. Partial Chemical Shift Assignments (δ) of ^{1}H and ^{13}C Resonances for the Different Monosaccharide Units in the MP-1 Fraction

H1	C1	H2	C2	H3	C3	residues[a]
5.34		4.03, 3.91		3.92		M^{11P}, M^{21P}
5.17[b]	100.7	4.00	78.18	3.80	71.15	M^{32}, M^{42}
5.07	102.1–103.2	3.97				M^{22P}
5.04[b]		3.96	70.54	3.78		M^{44}
5.02		4.11	70.12	3.90		
5.00[b]	98.1–99.45	3.93	79.28	3.81		M^{21}
4.97		3.92		3.76		M^{31}, M^{41}
4.95[b]		3.96	70.54	3.71		M^{33}
4.94	102.5–102.7	4.11	70.12	3.84		M^{22}
4.91		4.12				M^{43}

[a] According to assigment of Vinogradov et al. (*15*). [b] Major peaks.

This highly branched structure is in good agreement with the *S. cerevisiae* mannan branched structure proposed by Nakajima et al. (*13*). The structure found for the MP-1 fraction is also in agreement with the type linkages found by Waters et al. (*8*) for a high molecular weight mannoprotein extracted from wine, which suggests a highly branched polymer.

The wine mannoproteins consist of a mixture of populations covering different ranges of molecular weights. The fractionation of wine mannoproteins in two fractions showed that the highest molecular mass wine mannoproteins are a pure mannan associated with 10.3% protein, whereas the lowest molecular mass mannoproteins correspond to a glucomannan with 87.5% mannose and low protein content of 2.5%. The partial assignment of the higher molecular mass mannoprotein NMR signals revealed a branched structure similar to the structure of the intact *S. cerevisae* mannan.

LITERATURE CITED

(1) Feuillat, M.; Freyssinet, M.; Charpentier, C. L'elevage sur lies des vins blancs de Bourgogne II. Evolution des macromolécules: Polysaccharides et protéines. *Vitis* **1989**, *28*, 161−176.

(2) Vernhet, A.; Bellon-Fontaine, M.-N.; Brillouet, J.-M.; Roesink, E.; Moutounet, M. Wetting properties of microfiltration membrane: determination by means of the capillary rise technique and incidence on the adsorption of wine polysaccharide and tannins. *J. Membr. Sci.* **1997**, *128*, 163−174.
(3) Vernhet, A.; Pellerin, P.; Belleville, M.-P.; Planque, J.; Moutounet, M. Relative impact of major wine polysaccharides on the performance of an organic microfiltration membrane. *Am. J. Enol. Vitic.* **1999**, *50*, 51−56.
(4) Lubbers, S.; Leger, B.; Charpentier, C.; Feuillat, M. Effect Colloide-Protecteur d'extracts de parois de levures sur la stabilité tartrique d'une solution hydro-alcoolique modele. *J. Int. Sci. Vigne Vin* **1993**, *27*, 13−22.
(5) Gerbaud, V.; Gabas, N.; Laguerie, C.; Blouin, J.; Vidal, S.; Moutounet, M.; Pellerin, P. Effect of wine polysaccharides on the nucleation of potassium hydrogen tartrate in model solutions. *Chem. Eng. Res. Des.* **1996**, *74*, 782−789.
(6) Gerbaud, V.; Gabas, N.; Blouin, J.; Pellerin, P.; Moutounet, M. Effect des polysaccharides et des polyphénols du vin sur la cristallisation de l'hydrogénotartrate de potassium. *J. Int. Sci. Vigne Vin* **1997**, *31*, 65−83.
(7) Moine-Ledoux, V.; Perrin, A.; Paladin, I.; Dubourdieu, D. Premiers résultats de stabilisation tartrique des vins par addition de mannoprotéines purifiées (Mannostab). *J. Int. Sci. Vigne Vin* **1997**, *31*, 23−31.
(8) Waters, E. J.; Pellerin, P.; Brillouet, J. M. A *Saccharomyces* mannoprotein that protects wine from protein haze. *Carbohydr. Polym.* **1994**, *23*, 185−191.
(9) Moine-Ledoux, V.; Dubourdieu, D. An invertase fragment responsible for improving the protein stability of dry white wines. *J. Sci. Food Agric.* **1999**, *79*, 537−543.
(10) Dupin, I.; McKinnon, B. M.; Ryan, C.; Boulay, M.; Markides, A. J.; Jones, G. P.; Williams, P. J.; Waters, E. J. *Saccharomyces cerevisiae* mannoproteins that protect wine from protein haze: their release during fermentation and lees contact and a proposal for their mechanism of action. *J. Agric. Food Chem.* **2000**, *48*, 3098−3105.
(11) Lubbers, S.; Charpentier, C.; Feuillat, M.; Voilley, A. Influence of yeast cell walls on the behavior of aroma compounds in a model wine. *Am. J. Enol. Vitic.* **1994**, *45*, 29−33.
(12) Dufour, C.; Bayonoue, C. L. Influence of wine structurally different polysaccharides on the volatility of aroma substances in a model solution. *J. Agric. Food Chem.* **1999**, *47*, 671−677.
(13) Nakajima, T.; Ballou, C. E. Characterization of the carbohydrate fragments obtained from *Saccharomyces cerevisiae* Mannan by alkaline degradation. *J. Biol. Chem.* **1974**, *249*, 7679−7684.
(14) Saulnier, L.; Mercoreau, T.; Vezinhet, F. Mannoproteins from flocculating and non-flocculating *Saccharomyces cerevisiae* yeasts. *J. Sci. Food Agric.* **1991**, *54*, 275−286.
(15) Vinogradov, E.; Petersen, B.; Bock, K. Structural analysis of the intact polysaccharide mannan from *Saccharomyces cerevisiae* yeast using ^{1}H and ^{13}C NMR spectroscopy at 750 MHz. *Carbohydr. Res.* **1998**, *307*, 177−183.
(16) Llaubères, R. M.; Dubourdieu, D.; Villetaz, J.-C. Exocellular polysaccharides from *Saccharomyces* in wine. *J. Sci. Food Agric.* **1987**, *41*, 277−286.
(17) Pellerein, P.; Brillouet, J. M. Study of red wine polysaccharides fractionated by ion-exchange chromatography. *Vitic. Enol. Sci.* **1992**, *47*, 153−158.
(18) Albersheim, P.; Nevis, D. J.; English, P. D.; Karr, A. A method for the analysis of sugars in plant cell-wall polysaccharides by gas−liquid chromatography. *Carbohydr. Res.* **1967**, *5*, 340−345.
(19) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265−275.

Received for review March 4, 2002. Revised manuscript received July 23, 2002. Accepted July 25, 2002.

JF0202741