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Abstract

While they have many properties of interest in enology, the structure-function relationships of mannoproteins and the part played by their polysaccharide moiety are not yet well understood. Mannoproteins (MP) extracted with β-glucanase from a laboratory yeast strain (WT), two of its mutants (Mnn2 with unbranched N-glycosylated chains and Mnn4 without mannosyl-phosphorylation), and an enological strain (Com) were purified and thoroughly characterized. The protein moiety of the four MPs had the same amino acid composition. Glycosyl-linkage and net charge analyses confirmed the expected differences in mutant strain MPs. MP-Com had the highest mannose/glucose ratio followed by MP-WT/MP-Mnn4, and MP-Mnn2 (13.5>5.6≈5.2>2.2). The molar mass dependencies of R_g, R_h, and [η], determined through HPSEC-MALLS-QELS-Viscosimetry, revealed specific conformational properties of mannoproteins related to their nature of highly branched copolymers with two branching levels. It also clearly showed structural differences between MP-Com, MP-WT/Mnn4, and MP Mnn2, and differences between two populations within the four mannoproteins.

Keywords: Mannoproteins; HPSEC-MALLS-QELS-Viscosimetry; Structural Analysis; Wine; Hyperbranched biopolymers; Structure-function relationships.

1 Introduction

Mannoproteins represent an important family of wine polysaccharides. These proteoglycans, mainly located in the outer layer of yeast cell walls, are naturally released during wine fermentation and yeast autolysis in the aging on lees processes. In addition to the mannoproteins extracted during winemaking, the use of commercial preparations is authorized by the OIV (Organisation Internationale de la Vigne et du Vin) for specific purposes (Tartaric salts and/or protein stabilization). These commercial mannoproteins are extracted either through physico-chemical (heat, alkali, or sonication) or enzymatic methods. They must meet the specificities of the International Oenological Codex Resolution (Codex OIV-OENO 26–2004) and be composed of at least 60% in mass of polysaccharides, 70% of which must be mannose.

Mannoproteins account for 30-50% of the dry mass of *S. cerevisiae* cell wall, which itself represents 20-25% of the dry weight of the whole yeast cell (Aguilar-Uscanga & François, 2003; Kapteyn, Van Den Ende, & Klis, 1999). They are composed of an average of 85-90% glycans, mainly D-mannose, and 10-15% protein (Orlean, 2012). The mannan chains are linked to the protein through O and N-glycosidic bonds (**Figure 1**). N-glycans are composed of an inner oligosaccharide core and a long outer chain of 50 or more α-1,6-linked mannose residues, broadly branched by short chains of α-1,2- mannose in extension and α-1,3-mannose before the terminal (C. Ballou, 1976; Orlean, 2012). O-glycans are composed of linear structures of one to five α-1,2- and α-1,3-linked D-mannoses. Negatively charged mannosyl-phosphate groups can be attached to four different binding sites in the N-glycan, two in the core and two in the outer chain (**Figure 1**), and one position in O-linked oligosaccharides (Jigami & Odani, 1999). Mannoproteins are attached by their protein moiety to the inner layer of the cell wall, mainly composed of β-1,3-glucans and chitin through different pathways (Klis, Boorsma, & De Groot,

2006). Different cell-wall proteins, with different functions, have been identified (Reinhold et al., 1997; Teparić & Mrsă, 2013).

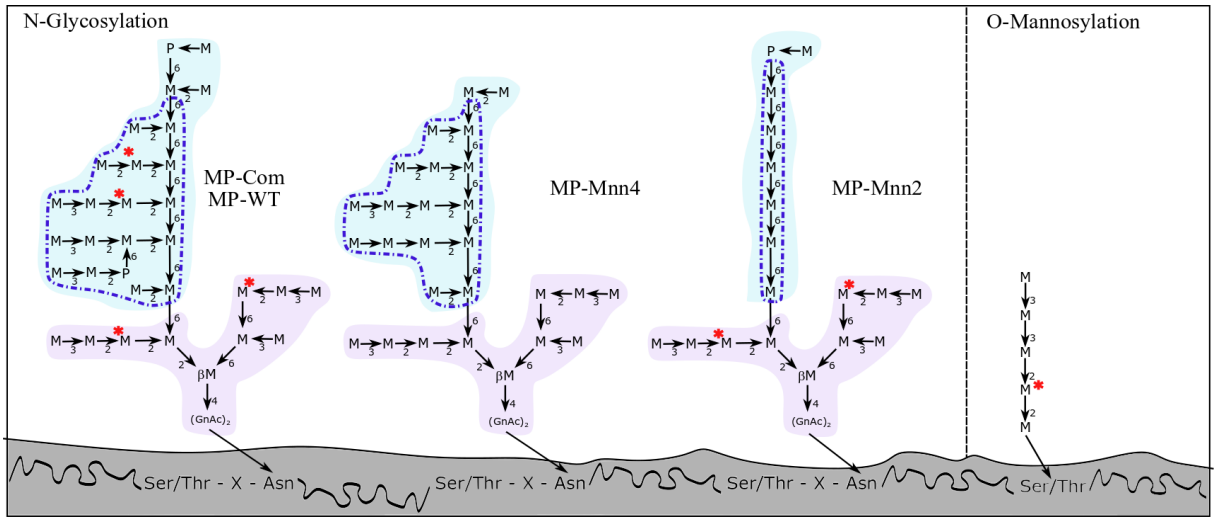

Figure 1: *Schematic representation of the N-linked and O-linked carbohydrate side-chains attached to the protein moiety of mannoproteins and the impact of ΔMnn4 and ΔMnn2 mutations on their structure. M: mannose residues; P: phosphate; regions in pink: core structure of the N-glycosylation; regions in light blue: outer chain structure of the N-glycosylated side chains of the mannoproteins from the corresponding yeast strains; region surrounded by a dashed violet line: repeated sequence of the outer chain of the N-glycosylation where the number of repetitions can vary. *: sites for the attachment of mannosyl-phosphate groups. The phosphorylation site in the O-linked structure is available for all MPs except MP-Mnn4. X: Any amino acid except Proline. Adapted from Corbacho and Hernandez (Corbacho, Olivero, & Hernández, 2005).*

Several works underline the various and positive functional properties of mannoproteins in enology (Caridi, 2006; Comuzzo, Tat, Battistutta, & Tasso, 2005): adsorption of mycotoxins (Ringot et al., 2005), stabilization against the crystallization of tartaric acid salts (Lankhorst et al., 2017; Lubbers, Leger, Charpentier, & Feuillat, 1993) and the formation of colloidal hazes or precipitates (Alcalde-Eon, García-Estévez, Puente, Rivas-Gonzalo, & Escribano-Bailón, 2014; Dufrechou, Doco, Poncet-Legrand, Sauvage, & Vernhet, 2015; Mekoue Nguela, Poncet-Legrand, Sieczkowski, & Vernhet, 2016; Poncet-legrand, Doco, Williams, & Vernhet, 2007; Waters, Pellerin, & Brillouet, 1994), improvement of organoleptic properties (mouthfeel, aroma perception, color) (Chalier, Angot, Delteil, Doco, & Gunata, 2007; Rinaldi, Gambuti, & Moio, 2012; Vidal et al., 2004), etc. These functional properties are mainly related to the

physicochemical interactions that develop between mannoproteins and wine constituents. However, these positive effects are variable and not fully understood yet. They are dependent on the wine composition, but more importantly, on the origin of the studied mannoproteins. Several studies indicate that the techno-functional properties of mannoproteins are dependent on their molar mass and the ratio between polysaccharide and protein moieties (Alcalde-Eon et al., 2014; Moine-Ledoux & Dubourdieu, 1999; Núñez, Carrascosa, González, Polo, & Martínez-Rodríguez, 2006; Poncet-legrand et al., 2007; Waters et al., 1994). However, neither the links between the structures of the polysaccharide and protein parts (composition, degree of branching, phosphorylation, etc.) and the properties of mannoproteins, nor their respective contributions to the latter, are yet clearly established.

The difficulty is that these structural features are expected to be heavily influenced by the provenance of the mannoprotein fractions: yeast strain used as a source and growth conditions, purification from wine, extraction from cell culture or lees through enzymes or physical treatments, etc. Thus, beyond the fact that only a few works have characterized the studied mannoproteins, the variability in their origin does not allow the identification of structure-function relationships (**Table 1**). Indeed, the release of mannoproteins in wines is related to yeast β-glucanase activities but other enzymatic activities may affect their initial structure. For example, yeast autolysis involves a huge variety of indigenous enzymes, the activities of which are no longer controlled by the dead yeast (Alexandre et al., 2001; Charpentier, 2010; Lurton, Segain, & Feuillat, 1989). In more controlled systems, enzymatic extractions are usually performed with commercial enzymes. Nevertheless, besides β-glucanases, other residual hydrolytic activities have been observed in these commercial cocktails, especially proteases and mannanases that can directly affect the mannoprotein structure (De Iseppi et al., 2019; Kath & Kulicke, 1999; Schiavone et al., 2014). As expected, mannoproteins extracted by physicochemical methods (heat, acid, and alkali extractions) also

differ in molar mass range and composition from those extracted through enzymes (De Iseppi et al., 2019; Kath & Kulicke, 1999; Moine-Ledoux & Dubourdieu, 1999; Núñez et al., 2006; Saunier, Mercereaua, & Vezinhet, 1991).

With the final objective to better understand the structure-function relationships of mannoproteins, we focused first on the role played by the structure of their polysaccharide part and by mannosyl phosphorylation. Indeed, the structure of the polysaccharide moiety may affect the conformation of the macromolecules and their interactions in solution. Mannosyl-phosphate groups, which carry a negative charge at wine pH, may be involved in electrostatic and ionic interactions with the other positively charged wine components such as pigments and are also possible sites for hydrogen bonding. To this end, a laboratory wild-type *S. cerevisiae* strain and two of its mutants, Mnn4 and Mnn2 genetically modified to not express mannosyl-phosphorylation and →2,6)-Man-(1→ branching linkages on the backbone of the N-glycosylation, respectively, were selected (**Figure 1**). An additional enological *S. cerevisiae* strain was also considered for comparison. The extraction method was designed to limit as much as possible the impact of yeast protease activities and thereby maintain to the utmost extent the native structure of mannoproteins. The first step, which is the subject of this article, was to extract and purify four mannoprotein pools with specific N-glycosylation and O-mannosylation structures from these strains and to thoroughly characterize them in terms of structure and conformation in solution. Our hypothesis is that the yeast strain and extraction procedure can strongly impact mannoprotein structures, whose conformations in solution are dependent their polysaccharide chains.

Extraction method	Reference	Extraction Conditions	Apparent M_w[1] (kDa)	Real M_w[2] (kDa)	Protein (mass %)	Polysacc. (mass %)	Mannose (molar %)	Glucose (molar %)	Other Sugars (molar %)	Phosphorus (mass %)
Purified after Alcoholic Fermentation or Autolysis	(Saunier et al., 1991)	Purified after AF	-	-	21	79	97.5	2.5	-	-
	(Waters et al., 1993)	Purified from red wine	60-23	-	4	96	78	13	9	-
	(Waters et al., 1994)	Purified from red wine	420	-	28.8	71.2	97.4	2.4	0.2 (GlcNAc)	-
	(Lubbers et al., 1993)	Purified/Fractionated after AF	-	-	10	90	87.7	12.3	ND	-
			-	-	12	86	83.3	16.7	ND	-
			-	-	5	90	87	13	ND	-
	(Dufrechou et al., 2015)	Purified from white wines	62	-	1.6	NS	88.8	2.6	8.5	-
	(Chalier et al., 2007)	Purified/ Fractionated after AF	225	-	11-13	NS	88-91	9-12	ND	-
			41	-	30-72	NS	65-68	32-35	ND	-
			10	-	-	NS	13	87	ND	-
	(Vidal et al., 2003) (Vernhet et al., 1996)	Purified/Fractionated from red wine according to their molecular charge	527	-	9.3	NS	91.1	5.1	3.9	-
			311	-	2.9	NS	95.9	3.6	0.6	0.34
			301	-	2.4	NS	89	3.6	6.8	0.43
			337	-	1.4	NS	92.4	ND	6.9	
			62	-	1.6	NS	88.8	2.6	8.5	0.13
			51	-	3.5	NS	97.1	1.9	1.7	
	(Gonçalves et al., 2002)	Purified from white wines	-	252-560	10.3	NS	100	ND	ND	-
			-	53.4	2.5	NS	87.5	2.6	9.8	-
Physico-chemical treatments	(Saunier et al., 1991)	120°C/90min	-	-	35	63.5	97.6	1.1	1.3 (GlcNAc)	1.5
	(Moine-Ledoux & Dubourdieu, 1999)	120°C/90min	-	-	4.2	93.8	92	8	ND	-
	(Núñez et al., 2006)	85°C/24h	-	-	31.3	66.1	61.3	4.9	ND	-
	(De Iseppi et al., 2019)	120°C/90min + 1min St	>250	-	7.9	92.1	84	16	ND	-
Enzymatic extractions	(Núñez et al., 2006)	Glucanex®	-	-	9.9	90.9	80	10.9	ND	-
	(De Iseppi et al., 2019)	Glucanex®	75-250	-	1.6	98.4	93.5	6.5	ND	-
		Zymolase®	150-250	-	1.2	98.7	69.4	30.6	ND	-
	(Moine-Ledoux & Dubourdieu, 1999)	Glucanex®	-	-	15	83.2	100	ND	ND	-

Table 1: *Literature data on the characterization of mannoproteins extracted by different methods. 1Mw measured through SDS-PAGE or Pullulan Calibration. 2Mw measured by SEC-MALS). NS: Results not shown, ND: not detected by the analysis, -: analysis not performed, St: Sonication time, AF: alcoholic fermentation in model media.*

2 Materials and methods

2.1 Extraction and purification of mannoproteins

2.1.1 Yeast strains and growth conditions

Four different *Saccharomyces cerevisiae* strains were used: on one hand, a commercial enological strain LMD47 provided by Lallemand SAS; on the other hand a wild-type BY4742 strain (MATα; ura3Δ0; leu2Δ0; his3Δ1; lys2Δ0), and its mutants ΔMnn4 (MATα; ura3Δ0; leu2Δ0; his3Δ1; lys2Δ0; YKL201c::kanMX4) and ΔMnn2 (MATα; ura3Δ0; leu2Δ0; his3Δ1; lys2Δ0; YBR015c::kanMX4). BY4742 and its mutant strains were obtained from EUROSCARF (European *Saccharomyces Cerevisiae* Archive for Functional Analysis). Yeast cells were first grown at 28°C into colonies in petri dishes on solid YEPD (1% yeast extract, 2% peptone, and 2% D-glucose with 2% agar-agar). Pre-cultures were prepared by inoculating 15 mL YEPD broth with a single colony and growing overnight (16-18 hours) at 28°C and under shaking. 5 mL of this first pre-culture were used to inoculate 500 mL of the second pre-culture. The yeast growth in the second pre-culture was monitored through the concentration of cells using a Coulter counter (Model Z2, Beckman-Coulter, Margency, France) fitted with a 100 µm aperture probe. This second pre-culture was used to inoculate 1 L of YEPD medium in a 5 L Erlenmeyer at a concentration of 10^6 cells.mL^{-1}. Cells were harvested at the beginning of the stationary growth phase, recovered by centrifugation, and inactivated at 70°C for 20 minutes. The inactivated biomass was dispersed in a YEPD medium added with 15% glycerol before being stored at -20 °C until use.

2.1.2 Mannoproteins extraction

Yeast cells (1g) were washed several times with deionized water before being dispersed in 4 mL of 0.2 M acetate buffer (pH 4.5). The extraction of mannoproteins was performed using a commercial Endo-β-1,3-glucanase enzymatic extract from *Trichoderma* sp. (E-LAMSE,

157 Megazyme, Ireland). This enzyme also contains a small residual Endo-β-1,6-glucanase activity
158 but no proteolytic activity (Schiavone et al., 2014). The enzyme solution (2mL) was added to
159 the cell dispersion and the hydrolysis took place at 40 °C under magnetic stirring for 17-18
160 hours. The reaction was stopped by enzyme inactivation at 70°C for 15 min. The suspension
161 was centrifuged (15000 g, 15min) and the supernatant was recovered in pre-treated dialysis
162 bags of 6-8 kDa size exclusion cut-off (Spectrum, Canada). Dialysis was performed in
163 deionized water for 3 days with water changes three times a day. Dialyzed extracts were
164 recovered and concentrated to a final volume of about 40 mL by evaporation at low temperature
165 (40 °C) under vacuum.

166 2.1.3 Purification of the Mannoproteins from the Enzymatic Extracts

167 The purification of the enzymatic extracts was performed through Anion Exchange
168 Chromatography assisted by an NGC Chromatographic System (BioRad, USA) equipped with
169 both diode array and conductivity detectors. Concentrated enzymatic extracts were diluted with
170 a 30 mM citrate-phosphate buffer (McIlvaine, 1921) at pH 3.5 until the target pH (3.5-3.6) was
171 reached. This buffer was prepared by mixing 69.65 mL of citric acid 0.1M and 30.35 mL of
172 Na_2HPO_4 0.2M before being 10-fold diluted. The solution was loaded in a prepacked HiPrep Q
173 Sepharose XL 20 mL column (GE Healthcare, USA) equilibrated with the same buffer (eluent
174 A). An unbound fraction was eluted with the equilibration buffer while a bound fraction (mainly
175 proteins) was recovered through a linear ionic strength gradient: from 100 % eluent A to 100
176 % eluent B (eluent A + NaCl 2 M) in 15 min. The unbound fraction, corresponding to the whole
177 mannoprotein content of the enzymatic extracts, was then further purified by ultrafiltration and
178 successive diafiltrations (up to a dilution factor 1000) to remove organic acids and salts.
179 Ultrafiltration was performed on a 30 kDa cut-off membrane composed of regenerated cellulose
180 from Millipore (Burlington, USA) using an Amicon® device (Burlington, USA) under the
181 pressure of 1.5 bar and stirring. The retentates (mannoprotein pools) were recovered, filtrated

on 0.45 μm hydrophilic membranes (mixed cellulose esters, Millipore), and concentrated at 10 $mg.mL^{-1}$ at 40 °C under vacuum. They were stored at -20 °C until further use. These mannoprotein pools are hereafter named according to the yeast strain from which they were purified: MP-Com, MP-WT, MP-Mnn4, and MP-Mnn2.

2.2 Characterization of the pools of mannoproteins.

2.2.1 Molecular weight distribution, static and dynamic molecular parameters.

The apparent molecular weight distribution of macromolecules in the enzymatic extracts, the non-retained fraction of the ion-exchange chromatography, and the purified pools were first determined by High-Performance Size-Exclusion Chromatography (HPSEC). HPSEC was performed using two serial Shodex OH-pak KB-803 and KB-805 columns (0.8 x 30 cm^2; Showa Denkko, Japan) coupled with an OH-pak KB-800P guard column. 50μL of the samples were eluted with a 0.45 μm filtered $LiNO_3$ 0.1 M solution (mixed cellulose ester membrane, Millipore) at a flow rate of $1mL.min^{-1}$. The system was equipped with ERC-7512 refractive index (Erma, Japan) and SPD-20A UV (280 nm, Shimadzu, Japan) detectors to distinguish between polysaccharides and proteins. The columns and UV detector were kept at room temperature while the refractive index detector was at 50 °C. The apparent molecular weight was calculated from a calibration curve established using 8 Shodex pullulan standards (P5: MW = 5 800 Da; P10: MW = 12 200 Da; P20: MW = 23 700 Da; P50: MW =48 000 Da; P100: MW = 100 000 Da; P200: MW = 186 000 Da; P400: MW = 380 000 Da; P800: MW = 853 000 Da; Showa Denko K.K.).

To determine the real molecular weight distribution and the static and dynamic parameters of the macromolecules, purified pools were also analyzed in an HPSEC system equipped with the same set of columns and the following set of detectors: a multi-angle (18 angles) laser light (wavelength 666 nm) scattering (MALLS) where one of the angles was

206 replaced by a Quasi-Elastic Light Scattering detector (QELS) (DAWN–Heleos II from Wyatt,
207 CA, USA), an on-line differential viscometer (VISCOSTAR II, Wyatt, CA, USA), an UV
208 detector (280 nm) (SPD-20A, Shimadzu, Japan) and a differential refractometer (666 nm,
209 Optilab T-Rex, Wyatt, Santa Barbara, CA, USA). Pools were dissolved in Milli-Q water at
210 4g.L^{-1} and centrifuged at 18 000 g/15 min before being eluted with a filtered (0.1 µm filter,
211 mixed cellulose ester, Millipore) solution (0.1 M $LiNO_3$ + 0.02% NaN_3) at a flow rate of 1
212 mL.min^{-1} and 40 °C. Data were analyzed using the Astra 6.1.2 software package. An average
213 refractive index increment value (dn/dc) of 0.150 mL.g^{-1} was used for the polysaccharide
214 moiety, n being the refractive index of the polysaccharide solution and c the polysaccharide
215 concentration in mass. The protein modifier method within the software was used to estimate
216 the respective molar masses of the protein and polysaccharide parts throughout the elution
217 profiles. To this end, the mass extinction coefficient of the protein part was determined from
218 UV absorbency measurements at 280 nm, performed with the whole mannoprotein pools. An
219 average value of 1.4 mL.mg^{-1}.cm^{-1} was determined and considered.

220 2.2.2 Neutral sugar analysis.

221 The neutral glycosyl-residues composition of the enzymatic extracts and mannoprotein
222 pools was determined by gas chromatography after polysaccharides hydrolysis with
223 Trifluoroacetic acid (Albersheim, Nevins, English, & Karr, 1967) and neutral sugar conversion
224 in their alditol acetate derivatives (Blakeney, Harris, Henry, & Stone, 1983). Inositol and allose
225 were added to the samples, after hydrolysis and before reduction and acetylation, as internal
226 standards for quantification (Doco, Quellec, Moutounet, & Pellerin, 1999). The
227 monosaccharides were identified through their retention time. GC-FID (Flame Ionization
228 Detector) was performed by a SHIMADZU GC-2010-Plus gas chromatography system using a
229 fused silica capillary column DB-225 (30m x 0.25 µm x 0.25 mm ID) (Agilent J&W, Santa
230 Clara, USA) with H_2 as carrier gas. The Student-Newman-Keuls test was applied to an analysis

of variance (ANOVA) to statistically cluster similar values of composition into the same group ($p<0.05$).

2.2.3 Composition of the Glycosyl-linkages

The covalent links involved between glycosyl-residues of the mannoproteins within each pool were measured by GC of the partially methylated alditol acetates following the Hakomori procedure of methylation (Hakamori, 1964). Per-methylated mannoproteins (1-2 mg) were submitted to pre-hydrolysis in 500 µL of formic acid 90% at 100 °C for one hour before being cooled to room temperature and dried under dry airflow at 40 °C. Pre-hydrolyzed polysaccharides were then submitted to another hydrolysis in 2M TFA before adding inositol as an internal standard. After their reduction with $NaBD_4$, monosaccharides were converted into their alditol acetate derivatives (Albersheim et al., 1967). Partially methylated alditol acetates were analyzed by GC–MS with a 30 m ·0.25 mm OV-1 column (temperature programming: 135 °C for 10 min, then 1.2 °C/min to 180 °C) with hydrogen (2 mL/min) as carrier gas. GC–MS was performed with a Hewlett–Packard HP-6890 GC coupled to a HP 5973 mass selective detector operating in the EI mode (70 eV, 34.6 lA, and m/z 50–650 uma) and under control of a HP Productivity ChemStation.

2.2.4 Protein analysis.

The Lowry method (Lowry, Rosebrough, Lewis Farr, & Randall, 1951) was used to assess the protein content of the enzymatic extracts before their purification through ion-exchange chromatography and ultrafiltration.

The protein mass percentage in the purified pools of mannoproteins, and the molar percentage of each amino acid residue in it, were quantified as follows. 10 mg of freeze-dried mannoproteins were dissolved in 0.5 mL of 6N HCl and hydrolyzed at 110 °C for 24 hours. After cooling the solution, 150 µL of norleucine solution at 2.5 M was added as an internal standard. Samples were then dried and washed twice with miliQ water and once with absolute

ethanol under dry airflow at 40°C. They were recovered in 750 µL of a commercial loading lithium citrate buffer at pH 2.2 and filtered with a 0.22 µm low protein binding filter (polyvinylidene fluoride - PVDF, Millipore). The quantification of the amino acids in samples was assessed by liquid chromatography with a Biochrom 30 analyzer (BIOCHROM 30, Cambridge, UK) using an ion-exchange column (Ultra-pac-8 lithium form; Amersham Pharmacia Biotech, Piscataway). As for the composition of the polysaccharide fraction, an ANOVA (SNK test, $p<0.05$) was applied to the amino acid residues composition.

2.2.5 Net charge density

The net charge density of the mannoproteins was measured by titration with a solution of a counter-charged polyelectrolyte using the Particle Charge Detector PCD-02 (Mütek, Germany) (Vernhet et al., 1996). The polyelectrolyte solutions used at a concentration of 10^{-4} N were the following: sodium polyethylenesulphonate as the anionic polyelectrolyte; and polydiallyldimethylammonium Chloride (Poly-Dadmac) as the cationic polyelectrolyte. The mannoproteins pools (10 $mg.mL^{-1}$) were diluted 100 times with a solution of 10^{-3} N NaCl. Their net charge was measured at 5 different pHs between 2 and 6, adjusted with 10^{-1} N HCl.

3 Results and Discussion

3.1 Production of mannoproteins from yeast biomass

Approximately 10 g of yeast cells (dry mass) from each of the four strains were treated with the commercial Endo-*β-1,3*-Glucanase. The yield of the enzymatic extraction and the composition of the enzymatic extracts (EEs) are given in **Table 2**. Mannose was the main component of all enzymatic extracts. However, all also had high amounts of glucose (especially that obtained from the Mnn2 strain) and protein (between 20 and 27%) when compared to most of the literature data (**Table 1**). For mannoproteins within the same range of molecular weight, the protein content usually represents between 4 to12% of the dry mass, this being highly

dependent on their origin (purified from wine, enzymatically extracted, and physically or chemically extracted). Glucose is usually present but in lower amounts than those found in the EEs. The HPSEC analysis with RI and UV detection showed different populations in the extracts (**Supplementary Data, Figure 1**). The main peak in RI detection, eluted with a maximum of around 15 min, can be attributed to mannoproteins from the ratio between the RI and UV signals within this range. For this peak, calibration with pullulans indicated Mw distributions ranging from 50 to 500 kDa, with a maximum intensity at 150 kDa for the four MPs. By contrast, populations eluted after 17 min were likely mainly proteins or other cell components with high absorbency at 280 nm.

	YC-Com	YC-WT	YC-Mnn4	YC-Mnn2
Total biomass used (g)	11	12	12	10
A	EE-Com	EE-WT	EE-Mnn4	EE-Mnn2
Yield of enzymatic extraction (mg EE/g YC)	119.2	157.7	124.5	122.4
Protein (mass %)	21.2 ± 3.5	24.8 ± 1.4	25.1 ± 2.0	26.9 ± 8.0
Polysaccharide (mass %)	62.9 ± 0.6	60.9 ± 7.3	65.9 ± 2.9	60.9 ± 8.9
Mannose (molar %)	80.8 ± 2.4	71.1 ± 4.6	71.2 ± 0.9	52.6 ± 1.2
Glucose (molar %)	17.4 ± 2.0	27.2 ± 4.5	27.7 ± 0.8	46.1 ± 1.4
B	IEC-Com	IEC-WT	IEC-Mnn4	IEC-Mnn2
Yield of MP production (mg MP/g YC)	101.2	116.3	105.9	103.1
Protein (mass %)	16.0 ± 1.1	18.0 ± 2.2	20.0 ± 1.3	20.0 ± 5.1
Polysaccharide (mass %)	59.5 ± 3.3	65.1 ± 0.7	62.2 ± 0.4	60.6 ± 4.2
Mannose (molar %)	82.3 ± 0.2	71.5 ± 0.5	72.3 ± 0.2	56.5 ± 0.2
Glucose (molar %)	16.2 ± 0.1	26.7 ± 0.6	26.2 ± 0.1	42.2 ± 0.1
Ratio Mannose/Glucose	5.1	2.7	2.8	1.3
C	MP-Com	MP-WT	MP-Mnn4	MP-Mnn2
Yield of MP production (mg MP/g YC)	59.3	86.4	79.4	69.1
Protein (mass %)	4.3 ± 0.3 b	4.0 ± 0.3 b	3.6 ± 0.3 b	5.4 ± 0.4 a
Polysaccharide (mass %)	85.0 ± 2.3 a	77.4 ± 1.8 b	82.6 ± 2.3 ab	82.3 ± 2.2 ab
Mannose (molar %)	91.2 ± 0.0 a	83.7 ± 0.8 b	82.4 ± 1.4 b	67.3 ± 0.2 c
Glucose (molar %)	6.8 ± 0.2 d	14.8 ± 0.3 c	15.8 ± 0.6 b	30.6 ± 0.3 a
Ratio Mannose/Glucose	13.5	5.6	5.2	2.2

Table 2: *Production yields (mg per g yeast cell biomass), protein contents, and neutral sugar compositions of the enzymatic extracts (A), of the enzymatic extracts after ion-exchange chromatography (B), and of the purified mannoprotein fractions (C). YC: yeast cells, EE: enzymatic extract, MP: Mannoproteins. Different letters indicate significant differences between samples for a given parameter (SNK's test for p-value < 0.05).*

Additional purification steps were applied to remove those constituents. The first step was ion-exchange chromatography. Both cation and anion-exchange chromatography were tested at different pHs, using either pH and ionic strength gradients for the elution. The best results were obtained using anion-exchange chromatography at a pH of 3.5, with an ionic strength gradient for the elution. These conditions allowed the recovery of an unbound fraction that included the mannoproteins (**Supplementary data, Figure 1**). The retained fraction was essentially composed of low molecular components and accounted for 15 to 25% of the enzymatic extract dry weight. This purification step induced a strong decrease of the UV adsorption within the low molecular weight range but both the protein and glucose contents remained high in the extracts, especially for the wild-type strain and its two mutants (**Table 2**). The exact nature of the retained fraction was not further investigated but its adsorption by anion-exchange chromatography at pH 3.5 was not expected for proteins, unless if they are very acidic. The removed components could be nucleic acids. An additional ultrafiltration step on 30 kDa membranes was performed to separate the low molecular weight components from the main population. It reduced the extraction yields to values within the range 6-9% (**Table 2**). These percentages agree with theoretical values: the yeast cell wall represents about 20-25% of the cell dry mass, and mannoproteins 30-40% of the cell wall so that 60 to 100 milligrams of mannoproteins per gram of dried yeast are expected (Aguilar-Uscanga & François, 2003; Kapteyn et al., 1999).

The analytical methods used explained 90% of mannoprotein dry weight (**Table 2**). The four MPs had similar neutral sugar content except for a slight but significant difference between MP-Com (85%) and MP-WT (77%). By contrast, the mannose/glucose ratio varied considerably from one MP to another. The highest ratio was found for MP-Com (13.5), followed by MP-WT (5.6) and MP-Mnn4 (5.21), and then MP-Mnn2 (2.2). These results indicated differences in the polysaccharide moiety between the mannoproteins of the

commercial and the wild-type yeast strains. They were also consistent with the expected impact of deletions for the mannoproteins extracted from the two mutant strains compared to those from the wild strain. The mutation of the Mnn4 strain results in the absence of mannosyl phosphate groups attached to the α-1,2-mannose branches of N-linked and O-Linked mannoproteins (**Figure 1**), and the mannose/glucose ratio was only slightly lower than that of MP-WT. By contrast, the mannoproteins from the Mnn2 mutant, expected to be devoid of α-1,2-mannan branches, exhibited a much lower mannose content, associated with a higher glucose content. The absence of mannan ramifications and the lower quantity of mannose residues in MP-Mnn2 may be the reason for the higher protein mass percentage observed in this pool (5.4%) when compared to those with less or non-affected polysaccharide structures (MP-Com: 4.29; MP-WT: 3.98; MP-Mnn4: 3.57), and the high glucose proportion in MP-Mnn2 might be a physiological response of the yeast cell to the depletion of the natural mannoprotein structure.

3.2 Amino acid composition of the protein part

The amino acid composition of the protein part of the four MPs is given in **Table 3**. They all had very similar amino acid compositions. Serine and threonine accounted together for about 35% of the total amino acids. These two residues are essential for both N-glycosylation or O-mannosylation attachments of the carbohydrate chains to the protein part (Orlean, 2012). Proline (~6%), alanine (~8%), glutamine/glutamic acid (~11%), valine (~8%), and asparagine/aspartic acid (~9%) represented about 42% of the total. Asparagin is another important amino acid, involved with serine and threonine in N-glycosylation (**Figure 1**) (Lehle & Bause, 1984). Other amino acids represented about 20% percent of the total, of which less than 2% were sulfur residues (cysteine, cysteic acid). Percentages of acidic, basic, hydrophilic, and hydrophobic residues were calculated from this composition (**Table 3**). For all four MPs,

25% of the protein moiety is composed of acidic and basic residues, almost 40% of hydrophobic residues, and about 38% of neutral hydrophilic residues. From these results, it could be concluded that the main differences between the studied mannoproteins are related to their polysaccharide part. There are only a few data on the amino acid composition of the mannoproteins, and the latter concern mannoproteins purified from red wine (Waters et al., 1994, 1993). Waters *et al.* found a higher % in serine and a lower % in aspartic acid, among other minor differences. As these mannoproteins were extracted from wine, these differences may be related to proteolytic activities.

Amino acid residue	MP-Com		MP-WT		MP-Mnn4		MP-Mnn2	
Cysteic acid	1.1 ± 0.8	a	1.1 ± 0.9	a	1.5 ± 0.5	a	1.2 ± 0.5	a
Alanine	8.5 ± 0.5	a	7.8 ± 0.3	a	8.0 ± 0.5	a	8.0 ± 0.4	a
Arginine	0.4 ± 0.1	b	1.0 ± 0.0	a	0.8 ± 0.3	ab	0.6 ± 0.2	ab
Aspartic acid	8.8 ± 0.2	c	9.1 ± 0.1	b	9.4 ± 0.0	a	9.2 ± 0.1	b
Glutamic acid	10.2 ± 0.6	c	10.8 ± 0.1	bc	11.1 ± 0.1	ab	11.6 ± 0.3	a
Glycine	3.3 ± 0.0	a	3.2 ± 0.4	a	3.2 ± 0.2	a	3.0 ± 0.1	a
Histidine	1.7 ± 0.1	a	1.6 ± 0.1	a	1.5 ± 0.2	a	1.3 ± 0.2	a
Isoleucine	3.4 ± 0.0	d	3.8 ± 0.1	c	4.0 ± 0.1	b	4.2 ± 0.0	a
Leucine	3.3 ± 0.2	a	3.3 ± 0.1	a	3.2 ± 0.3	a	3.2 ± 0.3	a
Lysine	3.8 ± 0.2	b	4.3 ± 0.1	a	3.9 ± 0.0	b	3.7 ± 0.1	b
Phenylalanine	1.1 ± 0.1	a	1.1 ± 0.1	a	1.2 ± 0.3	a	1.3 ± 0.3	a
Proline	6.4 ± 0.8	a	6.7 ± 0.4	a	6.0 ± 0.7	a	6.9 ± 0.4	a
Serine	12.8 ± 0.8	a	12.5 ± 0.5	a	13.0 ± 0.6	a	13.0 ± 0.5	a
Threonine	23.9 ± 0.8	a	23.5 ± 0.5	a	24.0 ± 1.2	a	23.7 ± 0.9	a
Tyrosine	1.6 ± 0.8	a	1.6 ± 1.1	a	1.0 ± 1.1	a	1.4 ± 1.0	a
Valine	9.3 ± 0.2	a	7.9 ± 0.2	b	8.1 ± 0.4	b	7.8 ± 0.3	b
Acidic residues	19.0 0.4	c	19.9 0.2	b	20.6 0.1	a	20.7 0.2	a
Basic residues	6.0 0.3	b	7.0 0.1	a	6.2 0.6	b	5.6 0.5	b
Hydrophilic residues	38.4 0.9	a	37.6 0.6	a	38.0 0.8	a	38.1 1.0	a
Hydrophobic residues	31.9 1.3	a	29.9 0.7	b	29.7 0.6	b	30.2 0.4	b

Table 3: *Composition of the protein moiety of the mannoprotein pools. Molar percentage of each amino acid residue. Percentage of acidic (glutamic and aspartic acids), basic (arginine, histidine, and lysine), hydrophilic (serine, threonine, and tyrosine), and hydrophobic (alanine, glycine, leucine, phenylalanine, proline, and valine) residues. Different letters indicate significant differences between samples for a given parameter (SNK's test for p-value < 0.05).*

3.3 Net charge density

The net charge of mannoproteins is pH-dependent and results from the presence of acidic phosphate groups in their polysaccharide moiety and of basic and acidic amino acid residues in their protein part. Since the overall amino acid compositions of all four MPs are very similar, differences between the four fractions are expected to be related to different amounts of mannosyl-phosphate units in their polysaccharide moiety. The highest phosphorylation levels are expected for MP-Com and WT. The genetic modification in Y-Mnn2 affects the number of possible sites for mannosyl-phosphorylation since the N-glycosylation backbone is linear and unbranched. However, there are other possible phosphorylation sites in the core of the N-linked and in the O-linked polysaccharide structure regulated by the Mnn4 phenotype (Olivero, Mañas, & M. Hernández, 2000; Orlean, 2012). Therefore, there is no mannosyl-phosphate in MP-Mnn4 so that its charge and isoelectric point (IP) will be governed by its protein moiety. The variation in the net charge density with pH is reported in **Figure 2** for the four MPs. The mannoprotein fractions had different IPs, which was consistent with different mannosyl-phosphate contents: the lowest was found for MP-Com (around 3.3), followed by MP-WT (3.5), MP-Mnn2 (3.7), and MP-Mnn4 (4.15). For pH below IP, the positive charge was weak whatever the fraction considered, in line with the low content in basic amino acids of their protein part (**Table 3**). For pH above their IP, MP-Com exhibited a higher negative charge than the others. This could reflect a higher phosphorylation degree by comparison to MP-WT. These results differ from previous ones obtained on different mannoproteins fractions purified from red wine (Vernhet et al., 1996). In this case, the purified mannoproteins all had an overall negative charge over a pH range of 2-9 and could be separated into three fractions by ion-exchange chromatography according to their charge (Vidal et al., 2003). Such separation was not possible in the present study. As already stated, this may be related to the presence of proteolytic activities during winemaking, along with β-glucanase ones. These proteolytic activities may be responsible for

different ratios between the mannosyl-phosphate groups on one hand and acidic and basic amino acid residues on the other hand.

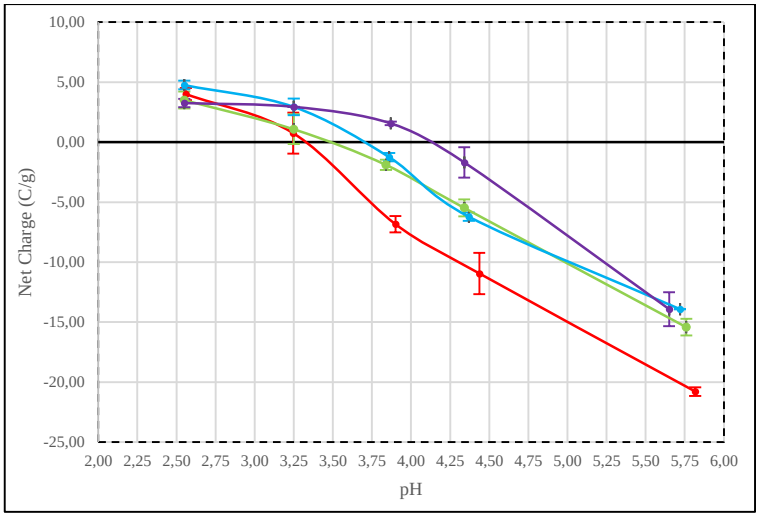

Figure 2: *The net charge density of the mannoprotein pools at different pHs. MP-Com (Red); MP-WT (Green); MP-Mnn4 (Violet) and MP-Mnn2 (Light Blue).*

3.4 Glycosyl Linkage Analysis

The molar percentages of glycosyl linkages for mannose and glucose in the purified mannoprotein pools are shown in **Table 4**. MP-Com, MP-WT, and MP-Mnn4 had similar compositions, with small differences among them. They had low amounts of →6)-Glc-(1→ from β-1,6-glucans chains that connect mannoproteins to the yeast cell wall. These linking structures are not fully hydrolyzed during the enzymatic extraction since the enzyme used has only a small residual β-1,6-glucanase activity (Schiavone et al., 2014). There was approximately three times less glucose associated with MP-Com by comparison to MP-WT and MP Mnn4. →2,6)-Man-(1→ residues are mainly related to the ramifications in the backbone of the N-glycosylation (**Figure 1**) and to the attachment sites of mannosyl-phosphate groups in the inner core and outer chains (Jigami & Odani, 1999). Their high proportions, associated with the low proportions of →6)-Man-(1→ (non-branched mannose residues in the backbone of N-glycosylated chains) and the presence of →2)-Man-(1→ and →3)-Man-(1→ residues belonging to the side chains of the outer-chain part and core of N-linked oligosaccharides and

to the linear structure of the O-linked oligosaccharides, highlighted the highly branched structure of the polysaccharide side chains of MP-Com, MP-WT, and MP-Mnn4 (L. Ballou, Hernandez, Alvarado, & Ballou, 1990). →6)-Man-(1→ and →3)-Man-(1→ units were found in higher proportions (between 33 et 42% higher) in MP-Com than in MP-WT and MP-Mnn4. This was associated with lower proportions of →2)-Man-(1→ (between 13 to 15%). This suggested differences in the branching patterns of the N-glycosylated chains between these mannoproteins.

		MP-Com	MP-WT	MP-Mnn4	MP-Mnn2
Glycosyl Residue	Linkage				
2,3,4,6-Glucose	Glc-(1→	0.7	1.5	1.5	4.8
2,3,4-Glucose	→6)-Glc-(1→	2.2	5.7	6.4	20
2,4-Glucose	→3,6)-Glc-(1→	-	-	1.6	3.7
Total Glucose (% molar)		2.8	7.3	9.5	28.5
2,3,4,6-Mannose	Man-(1→	29.7 (30.6)	30.6 (33.0)	29.5 (32.6)	23.4 (32.7)
2,3,4-Mannose	→6)-Man-(1→	1.2 (1.2)	0.7 (0.8)	0.8 (0.9)	20.1 (28.1)
2,4,6-Mannose	→3)-Man-(1→	18.9 (19.4)	11.4 (12.3)	10.3 (11.4)	6.2 (8.7)
3,4,6-Mannose	→2)-Man-(1→	21.3 (21.9)	23.5 (25.4)	22.8 (25.2)	18.3 (25.6)
2,4-Mannose	→3,6)-Man-(1→	0.6 (0.6)	0.4 (0.4)	0.6 (0.7)	1.3 (1.8)
3,4-Mannose	→2,6)-Man-(1→	25.2 (25.9)	25.8 (27.8)	26.5 (29.3)	1.2 (1.7)
3-Mannose	→2,4,6)-Man-(1→	0.3 (0.3)	0.2 (0.2)	0.3 (0.3)	0.9 (1.3)
Total Mannose (% molar)		97.2 (100.0)	92.7 (100.0)	90.5 (100.0)	71.5 (100.0)
Branching Degree (DB) (%)		56	60	62	13

Table 4: *Molar percentage of the glycosyl linkage residues in the polysaccharide moiety of each MP. Values in parentheses represent the molar percentages only in mannosyl linkage residues. The branching degree of the mannan chains of the polysaccharide moiety was calculated according to Hölter et al. (Hölter, Burgath, & Frey, 1997).*

As expected, the strongest differences were evidenced between MP-Mnn2 and the three other mannoproteins. MP-Mnn2 had much lower contents of branched →2,6)-Man-(1→) residues and much higher amounts in →6)-Man-(1→, which reflects the lack of branching of the outer chain of the N-glycosylated oligosaccharides. The branched residues still present in MP-Mnn2 likely belong to the core structure, unaffected by the gene deletion. Molar percentages of linear →2)-Man-(1→ and →3)-Man-(1→, similar to the other three MPs, could

be an indication that mannoproteins in this yeast strain have higher proportions in O-linked than N-linked side chains. As already stated, MP-Mnn2 also had a higher proportion of glucose than the other mannoproteins, which is mostly related to →6)-Glc-(1→ residues.

3.5 Molar mass distribution and molecular properties

The HPSEC-MALS chromatograms of the four mannoprotein pools are shown in **Figure 4**. The elution profiles (RI and UV) are represented along with the molar mass (M_w) profile calculated from the SLS (Static Light Scattering) measurements. All MP pools presented populations of different sizes and proportions. The HPSEC profiles showed two populations for MP-com: a small population in terms of amount with a high hydrodynamic radius (R_h) and average molar mass, thereafter named population 1, and a predominant population of lower hydrodynamic radius and average molar mass, thereafter named population 2. A third population, eluted last, was also evidenced for MP-WT, MP-Mnn2, and MP-Mnn4. The proportions of these different populations within each mannoprotein pool, along with the static (M_w, M_n, R_g) and dynamic ([η], R_h) average parameters of populations 1 and 2, are summarized in **Table 5.** The third population detected in MP-WT, MP-Mnn2 and MP-Mnn4 only represented a low mass percentage and was mainly co-eluted with population 2. Thus, analytical results were unreliable and won't be further discussed. The relative UV signal by comparison to the RI one also indicated higher protein/polysaccharide ratios for the mannoproteins in population 1 compared to those in population 2. Assuming for the protein part a close mass extinction coefficient over the whole distribution, and considering dn/dc value of 0.185 and 0.146 mL/g for proteins (Zhao, Brown, & Schuck, 2011) and polysaccharides (Ioan, Aberle, & Burchard, 2000; Denis Renard, Lavenant-Gourgeon, Ralet, & Sanchez, 2006; Rolland-Sabaté, Mendez-Montealvo, Colonna, & Planchot, 2008), respectively, it was possible to calculate for the two populations of each MP pool a protein/polysaccharide ratio and the respective molar mass of the protein and polysaccharide parts (**Figure 4 and Table 5**).

	MP-Com	MP-WT	MP-Mnn4	MP-Mnn2
Total Pool				
Peak Limits (min)	13 – 18.1	13 – 18.8	13 – 19.2	13 – 20
Mass Fraction (%)	100	100	100	100
Mass Recovery (%)	81	82	100	87
Protein Mass (%)	3.4	3.9	3.9	5.6
Total Mw (x10^5 g.mol-1)	4.6 ± 0.0%	13.0 ± 0.1%	11.9 ± 0.1%	17.3 ± 0.1%
Total Mn (x10^5 g.mol-1)	2.8 ± 0.0%	5.0 ± 0.1%	4.2 ± 0.1%	6.2 ± 0.2%
PdI (Mw/Mn)	1.6 ± 0.1%	2.6 ± 0.1%	2.8 ± 0.2%	2.8 ± 0.3%
Protein Mw (x10^5 g.mol-1)	0.2 ± 0.9%	0.8 ± 0.5%	0.7 ± 0.5%	1.3 ± 0.4%
Protein Mn (x10^5 g.mol-1)	0.1 ± 0.3%	0.2 ± 0.3%	0.1 ± 0.3%	0.3 ± 0.4%
Protein PdI (Mw/Mn)	2.1 ± 0.9%	4.8 ± 0.6%	5.1 ± 0.6%	4.4 ± 0.5%
R_g (nm)	13.8 ± 0.3%	32.9 ± 1.3%	34.9 ± 1.3%	45.7 ± 0.5%
R_h (nm)	12.1 ± 0.1%	15.8 ± 0.2%	15.3 ± 0.1%	18.0 ± 0.2%
[η] (ml.g^{-1})	30 ± 0.3%	30 ± 0.4%	30 ± 0.3%	33 ± 0.3%
Population 1				
Peak Limits (min)	13 – 14.5	13 – 14.4	13 – 14.7	13 – 14.4
Mass Fraction (%)	6.6	12.1	15.1	14.6
Protein Mass (%)	5.2	6.7	6.3	8.3
Total Mw (x10^5 g.mol^{-1})	21 ± 0.0%	65 ± 0.1%	53 ± 0.1%	74 ± 0.1%
Total Mn (x10^5 g.mol^{-1})	19 ± 0.0%	57 ± 0.1%	41 ± 0.1%	69 ± 0.1%
PdI (Mw/Mn)	1.1 ± 0.0%	1.1 ± 0.1%	1.3 ± 0.1%	1.1 ± 0.1%
Protein Mw (x10^5 g.mol^{-1})	1.1 ± 1.6%	4.5 ± 0.5%	3.5 ± 0.5%	6.5 ± 0.4%
Protein Mn (x10^5 g.mol^{-1})	1 ± 1.1%	3.7 ± 0.5%	2.5 ± 0.5%	5.7 ± 0.3%
Protein PdI (Mw/Mn)	1.2 ± 2.0%	1.2 ± 0.7%	1.4 ± 0.7%	1.1 ± 0.5%
R_g(nm)	24 ± 0.0%	34 ± 0.3%	34 ± 0.3%	38 ± 0.5%
R_h (nm)	24 ± 0.3%	34 ± 0.2%	32 ± 0.1%	38 ± 0.1%
[η] (ml.g^{-1})	44 ± 0.9%	39 ± 0.7%	42 ± 0.4%	46 ± 0.4%
Population 2				
Peak Limits (min)	15 – 18.1	15 – 17	15 – 17.5	15 – 17
Mass Fraction (%)	92.8	66.6	71.7	51.9
Protein Mass (%)	3.3	3	3	5.2
Total Mw (x10^5 g.mol^{-1})	3.4 ± 0.0%	5.4 ± 0.1%	4.5 ± 0.1%	8 ± 0.2%
Total Mn (x10^5 g.mol^{-1})	2.7 ± 0.0%	4.5 ± 0.1%	3.7 ± 0.1%	5.7 ± 0.2%
PdI (Mw/Mn)	1.3 ± 0.1%	1.2 ± 0.1%	1.2 ± 0.2%	1.4 ± 0.3%
Protein Mw (x10^5 g.mol^{-1})	0.12 ± 1.6%	0.2 ± 0.3%	0.2 ± 0.3%	0.5 ± 0.3%
Protein Mn (x10^5 g.mol^{-1})	0.08 ± 1.1%	0.1 ± 0.2%	0.1 ± 0.3%	0.3 ± 0.3%
Protein PdI (Mw/Mn)	1.4 ± 2.0%	1.4 ± 0.4%	1.3 ± 0.4%	1.7 ± 0.4%
R_g (nm)	13 ± 0.5%	25 ± 0.7%	25 ± 1.3%	37 ± 0.3%
R_h (nm)	11 ± 0.1%	13 ± 0.1%	12 ± 0.1%	16 ± 0.1%
[η] (ml.g^{-1})	29 ± 0.3%	30 ± 0.2%	29 ± 0.2%	35 ± 0.2%

Table 5: *Average molecular parameters of the two main populations observed in the HPSEC-MALS profiles of the four mannoprotein pools (MP-Com, MP-WT, MP-Mnn2, and MP-Mnn4). Mw: Molecular weight in mass, Mn: Molecular weight in number, PdI: Polydispersity Index, Rg: Radius of Gyration, Rh: Hydrodynamic radius, [η]: intrinsic viscosity.*

The predominant populations in the four mannoprotein pools (populations 2) had average M_w ranging between $3.4x10^5$ (MP-Com) and $8x10^5$ (MP-Mnn2) $g.mol^{-1}$, and polydispersity indexes between 1.2 and 1.4. The protein moiety represented between 3 to 5% in mass of the whole macromolecules. Population 1 only represented 6.6% of MP-Com but between 12 to 15 % of the other mannoprotein pools. It had much higher M_w than the population 2 (from 6 in MP-Com to 9-12 times higher in MP-WT, Mnn2, and Mnn4), associated with lower polydispersity indexes in all MP pools but MP-Mnn4. Populations 1 also had higher protein contents (between 5 to 8% in mass) than populations 2. These different proportions indicate that populations 1 are not related to aggregates of macromolecules belonging to population 2. The hydrodynamic radius of these high molecular weight populations was about twice higher than those of population 2. Although the differences in R_h between the two populations in the mannoprotein pools were relatively small, they were associated with substantial differences in molecular weight. Average M_w, determined by SLS (Static Light Scattering), ranged from 460kDa (MP-Com) to 1 730 kDa (MP-Mnn2), which were higher than those indicated by calibration with pullulans (maximum values around 150kDa for all mannoprotein pools, **Supplementary Data, Figure 1**), as expected for branched structures. The real molecular weight distributions in the four MPs were also different than those observed in the literature. While MP-Com oscilated from 100 - 4 000 kDa the other three MP pools fluctuated between 300 kDa and 11 000 kDa (**Figure 4**). Whereas mannoproteins purified from white wines (molecular weight also determined by MALLS) ranged from 30 to 300 kDa (Gonçalves et al., 2002). When testing different methods for mannoprotein extraction from brewery and bakery *S. cerevisiae* strains, Kulicke & Kath observed that the highest molecular weights (M_w of 410 kDa determined through HPSEC-MALS) were obtained when mannoproteins were extracted with commercial β-glucanases completely free of protease activity (Kath & Kulicke, 1999), as in the present study.

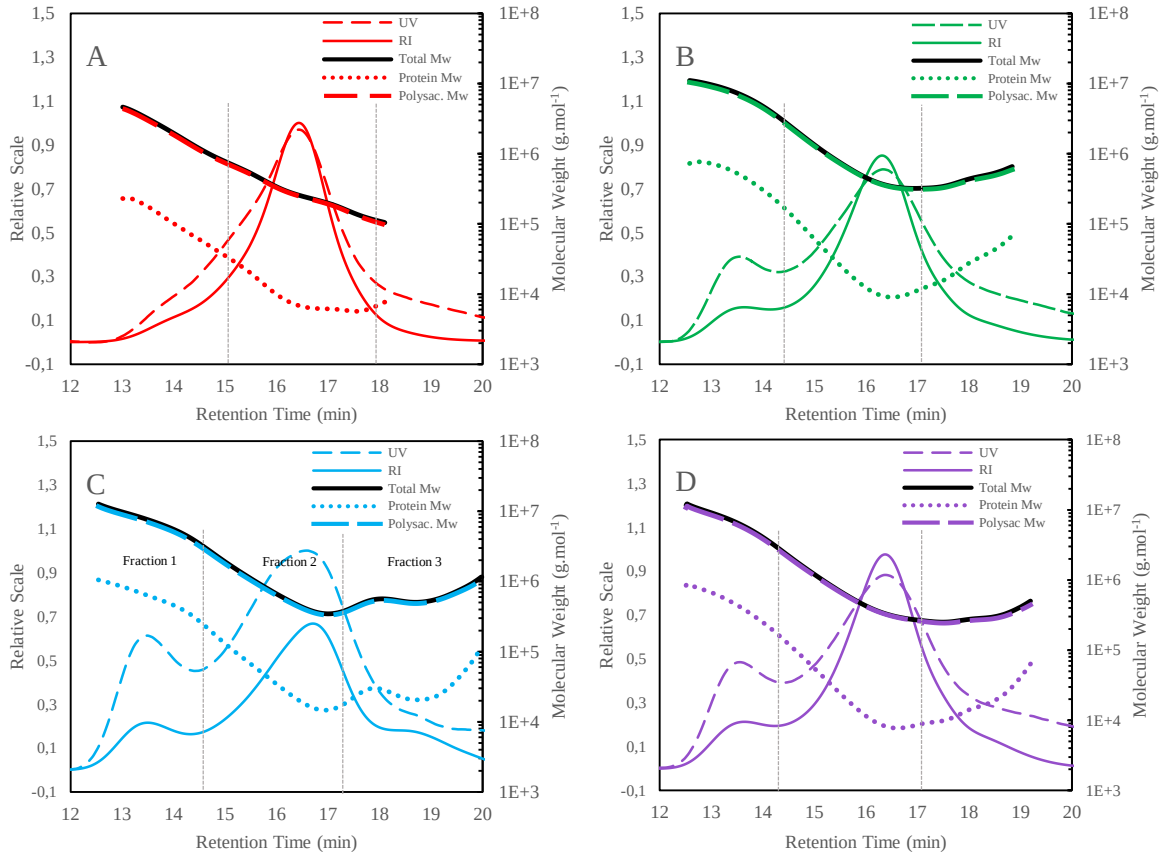

Figure 4 *: HPSEC-MALS profiles of the four pools of mannoproteins. A) MP-Com (Red), B) MP-WT (Green), C) MP-Mnn4 (Violet), and D) MP-Mnn2 (Light Blue). UV and RI detection profiles are presented on a relative scale. In the secondary axis, three molar mass profiles are presented for each mannoprotein: total molar mass (black lines); molar mass of the protein moiety (colored dotted lines); molar mass of the polysaccharides moiety (colored dashed lines).*

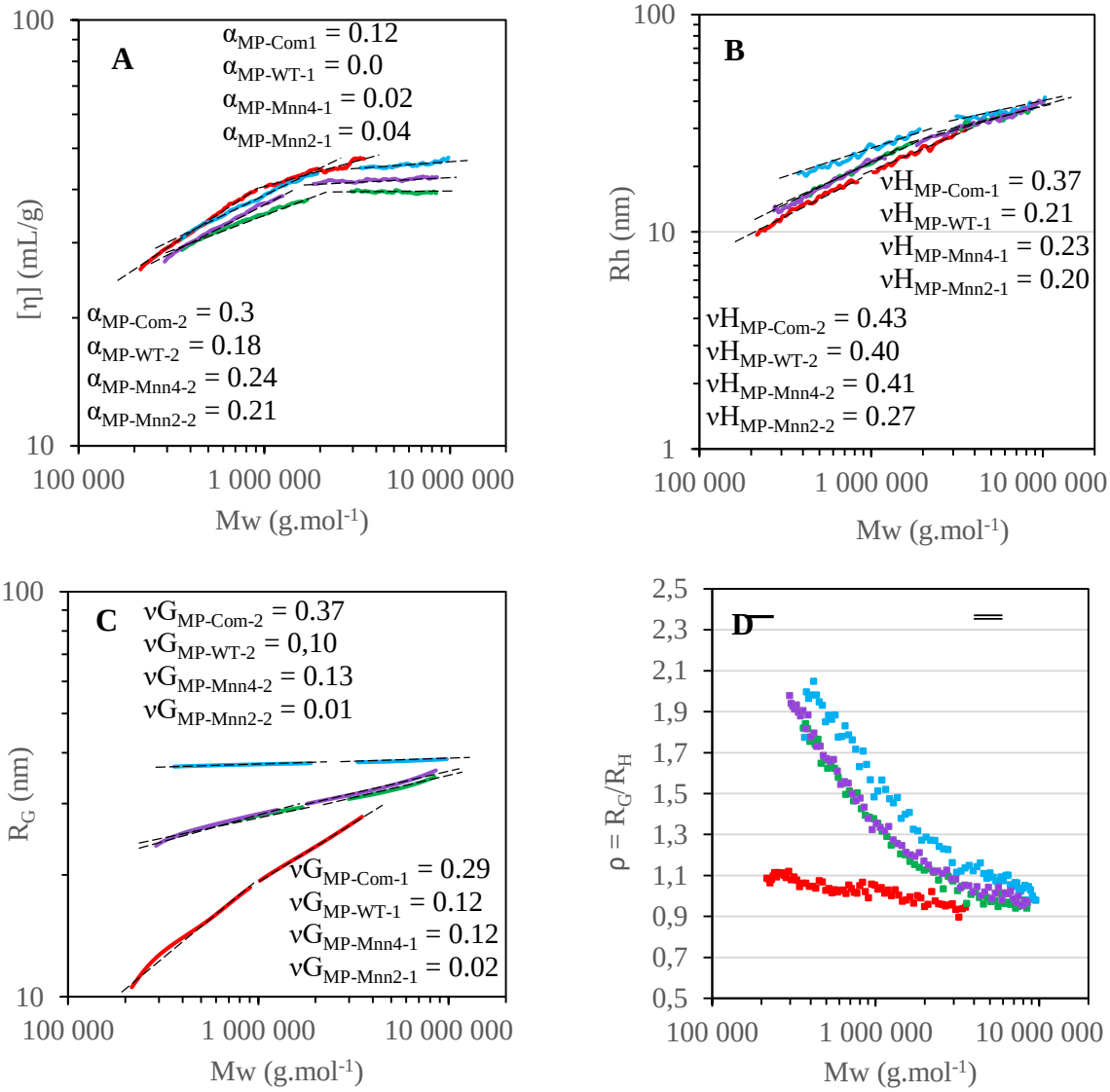

Figure 5: ***A****) Mark-Houwink-Sakurada (MHS) plot showing the Intrinsic Viscosity as a function of the molar mass (Mw) and the* α *coefficients (*$[\eta] = K_\alpha M_w^{\alpha}$*) for the 2 main populations (1 and 2) of each MP.* ***B****) Radius of gyration (Rg) as a function of the molar mass (Mw) and* νG *coefficients (*$R_g = K_g M_w^{\nu G}$*) for the 2 main populations (1 and 2) of each MP.* ***C****) Hydrodynamic Radius (*R_h*) as a function of the molar mass (Mw) for the main fractions of each pool of MPs and the average* νH *coefficient (*$R_h = K_h M_w^{\nu H}$*).* ***D****) The structural parameter* ρ *(*$\rho = R_g/R_h$*) as a function of the molar mass (Mw) for the two main fractions of the four pools of mannoproteins. MP-Com (Red); MP-WT (Green); MP-Mnn4 (Violet) and MP-Mnn2 (Light Blue).*

The Intrinsic Viscosity [η], radius of gyration (R_g), hydrodynamic radius (R_h), and ρ parameter ($\rho = R_g/R_h$) also known as asymmetry or anisotropy parameter) are plotted as a function of the molar mass M_w in **Figures 5A, 5B, 5C, and 5D**, respectively. For monodisperse polymers of sufficiently high molar mass and at infinite dilution, [η], (R_g), and R_h are related to the M_w through a simple power law:

$$[\eta] = K_\alpha M_w^{\alpha} \quad (1)$$

$$R_g = K_g M_w{}^{\nu G} \quad (2)$$

$$R_h = K_h M_w{}^{\nu H} \quad (3)$$

where α, νG, and νH are the corresponding hydrodynamic coefficients and K_α, K_g, and K_h the corresponding constants. The values of α, *νG*, and ν*H* provide some insights into the shape, anisotropy, and solvation of the macromolecules in the medium (good solvent or not) (Burchard, 1999). The ρ parameter is dependent on the polymer architecture and conformation (**Supplementary data, Table 2**). Since the polymer flexibility, polydispersity, density, and homogeneity affect directly R_g, they also influence the ρ values.

The Mark-Howink-Sakurada plots ([n] = f(M_w)) of the four mannoprotein pools evidenced a change in slope from population 2 to population 1 (**Figure 5A**). The impossibility to resume the whole curve in one power law (and consequently, one α coefficient) highlighted structural differences between these two populations (Ioan et al., 2000; Lopez-Torrez, Nigen, Williams, Doco, & Sanchez, 2015). α values go from 0 (corresponding to a sphere-shape population) to 1.8 (rod-like shape). Flexible polymers usually have intermediate α values (0.5-0.8), dependent on the interactions between the macromolecules and the solvent (Burchard, 1999). α evolves inversely to their degree of branching and values below 0.5 indicate a hyperbranched polymer structure. Also, α decreases as the M_w increases for macromolecules with a branching degree higher than 0.5. The α values of population 2 in the four pools varied between 0.17 and 0.3. Such coefficients correspond to hyperbranched polymers with a homogeneous repartition through the hydrodynamic volume and suggest that most of the volume of the macromolecules remain well solvated and loosen, yet with an undefined shape. α values below 0.5 were also found upon a wide range of molecular weight for Acacia Seyal and Senegal Gums (Apolinar-Valiente et al., 2019; Lopez-Torrez et al., 2015), which are other natural hyperbranched polysaccharides. A clear decrease in α was observed between population 2 and population 1 for all MPs, the [η] becoming independent on Mw (α ~ 0) for MP-WT,

Mnn4, and Mnn2. This decrease suggests a difference in conformation and an increase in density between the two populations due to an increase in the branching degree and/or length of the chains (Ioan, Aberle, & Burchard, 1999; Rolland-Sabaté, Colonna, Mendez-Montealvo, & Planchot, 2007; Rolland-Sabaté et al., 2008). Ioan et al. (1999) also observed α values close to zero with glycogens, which are natural hyperbranched polysaccharides (Ioan et al., 1999). Although the branching degree of its polysaccharide moiety was much smaller (**Table 4**), α values found for both MP-Mnn2 populations were similar to those determined for MP-WT and MP-Mnn4. Differences in branching degree calculated by glycosyl-linkage analysis between these mannoproteins are related to the N-glycosylated chains. They do not reflect the degree of branching of the protein part by O and N-glycan carbohydrate chains. These similar α values suggest that the molar mass dependence of $[\eta]$ is mainly governed by the degree of branching of the protein moiety.

For the same Mw, differences were observed in the R_g and R_h values of the mannoproteins (**Figure 5B and C**). Both the *vG* (around 0.3) values obtained for the two populations of MP-Com (Red) were consistent with a sphere-like conformation (Ioan, 1999). The *vH* values around 0.4 found on the whole M_w distribution (between the sphere and the random coil theoretical values) indicated some anisotropy of the hydrodynamic volumes of the macromolecules (**Figures 5B and C**). The *vH* values determined within the "low" M_w range for MP-WT and MP-Mnn4 were closed to that found for MP-Com (~ 0.4), whereas it was lower for MP-Mnn2 and close to the theoretical one for a sphere shape (~ 0.3). Contrarily to what was observed with MP-Com, *vH* values decreased between the lowest and the highest molar mass populations, by a factor of 2 for MP-WT and Mnn4 and to a lower extent for MP-Mnn2. On the other hand, the *vG* coefficients determined for the two populations of the WT, Mnn2, and Mnn4 mannoproteins were much lower than those found for MP-Com: they varied between 0.1 and 0.13 for the MP-WT and MP-Mnn4 and dropped to values between 0.012 and 0.017 for the

MP-Mnn2. These quite low values indicated a particular behavior in terms of molecular mass dependence of the radius of gyration and were much lower than those usually reported for other branched/hyperbranched polysaccharides (between 0.27 and 0.5) (Lopez-Torrez et al., 2015; Rolland-Sabaté et al., 2007, 2008). They were also lower than that theoretically expected for spherical particles of uniform density ($\nu G = 1/3$). Mannoproteins are heteropolymers consisting of a proteic moiety that carries polysaccharide side chains. They may differ within the same population in terms of the number, size, and structure of these carbohydrate chains (O and N-glycosylated chains), as well as in the structure of the later. These macromolecules are thus hardly comparable to literature data established with synthetic polymers with well-defined compositions and architectures or even with other branched polysaccharides. They can be explained if the increase in M_w throughout the distribution is associated with an increase in the branching density by carbohydrate side chains (number of branching points) and/or chain length (Burchard, 1999; Ioan et al., 2000; Rolland-Sabaté et al., 2008). Studying synthetic copolymers containing different and controlled amounts of long-chain branching, Auhl et al. observed a deviation from the power law that increased with the molar mass, with R_g values that reached a plateau (R_g independent of M_w) at high molar masses, behavior that clearly indicated long chain branching in this specific case (Auhl et al., 2004). A similar dependence was found for statistically branched homopolymers with a branching content increasing with molar mass (Gabriel & Münstedt, 2002; Jackson, Chen, & Mays, 1996) and for dextrans (Ioan et al., 2000). Such an increase in branching density between the low and high molar mass populations for MP-WT, Mnn4, and Mnn2, resulting in an increase in molecular density, is consistent with the changes observed in α values between the two populations of these mannoproteins and with the decrease in νH: when associated with an increase in branching, the increase in M_w does not induce the strong changes in R_h that would be observed for less branched polymers.

563 The ρ parameter found for MP-Com and the high molar mass population in the three
564 other MP pools varied with the M_w between 1.1 and 0.9, which is consistent with dendrimeric
565 (0.977) or regular star (1.079-1.33) structures (**Supplementary Data, Table 2**), and is within
566 the range of ρ found for other branched polysaccharides such as amyloses, dextrans, glycogens,
567 and some fractions of Acacia Senegal and Acacia Seyal gums (Ioan et al., 1999; D Renard et
568 al., 2014; Rolland-Sabaté et al., 2008). By contrast, for population 2 of MP-WT/Mnn4/Mnn2,
569 a strong decrease of ρ from 2 to 1.1 was observed when the M_w increased. Theoretical ρ-values
570 above 1.3 are usually related to linear random coil structures, which do not correspond to
571 mannoproteins as confirmed by the power law coefficients obtained from MHS, R_gxM_w, and
572 R_hxM_w plots and the glycosyl-linkage composition analysis. In this specific case, this is related
573 to the molar mass dependence of R_g and R_h, attributed to an increase in branching density by
574 carbohydrate side chains with the M_w and/or increasing side chain length.
575 Overall, these results clearly show structural differences between MP-Com, MP-
576 WT/Mnn4, and MP Mnn2, and differences between the "low" and "high" molar mass
577 populations within the four pools. Any comparison with other biopolymers was limited by the
578 fact that there is no consistent literature data on α, νG and νH coefficients of mannoproteins,
579 and that their composition as a natural copolymer is quite specific.

580 **4 Conclusion**

581 The extraction and purification methods applied in this study made it possible to obtain
582 pools of mannoproteins from 4 yeast strains: a laboratory wild-type strain (BY4742), two of its
583 mutants (ΔMnn4 and ΔMnn2, in which genetic deletions affect the polysaccharide moiety), and
584 a commercial enological strain (LMD47) as control. The differences in the molecular
585 characteristics of mannoproteins caused by genetic deletions (ΔMnn2 and ΔMnn4) on the strain
586 BY4742 were confirmed and differences were also observed between the laboratory and the
587 enological strains.

588 The composition of the protein and polysaccharide moieties of MPs showed that the protein
589 moiety between the four MPs pools are similar and that the main difference comes from the
590 polysaccharide part. As expected, differences from the normal polysaccharide structure of MP-
591 Mnn2 and MP-Mnn4 when compared to MP-WT altered their net charge balance – usually
592 ruled by both the protein and polysaccharide moieties – and the molecular conformation of MP-
593 Mnn2. These characterizations also evidenced differences in structure, composition, and
594 properties between MP-Com and MP-WT. Moreover, the comparison of the mannoproteins
595 obtained in this work with the literature data, in terms of net charge, apparent and/or real
596 molecular weight distributions, and protein content confirmed that the extraction method
597 developed has little effect on the native structure of mannoproteins. In fact, mannoproteins
598 purified from wine or extracted by the use of enzymatic cocktails (such as Zymolase® or
599 Glucanex®) are also submitted to proteolytic and/or mannolytic activities and might not be
600 representative of their structure in the yeast cell wall. The analysis of the static and dynamic
601 parameters provided by HPSEC-MALLS revealed very specific conformational properties of
602 mannoproteins related to their nature of highly branched natural copolymers with two branching
603 levels: a protein more or less branched by irregular polysaccharides chains, which in turn are
604 also more or less branched. However, any comparison with other sources of mannoproteins was
605 limited by the fact that there is no literature data on α, νG and νH coefficients for these
606 macromolecules.
607 The four mannoprotein pools obtained and characterized will be used to study how the
608 structure of the polysaccharide moiety, which affects the structure and conformation of the
609 macromolecule, and mannosyl-phosphate groups, which carry a negative charge at wine pH,
610 impact their interactions with wine constituents and their functional properties in enology.

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