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Structural characteristics of *Saccharomyces cerevisiae* mannoproteins: impact of their polysaccharide part

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

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

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**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).
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. [1]
<table>
<thead>
<tr>
<th>Extraction method</th>
<th>Reference</th>
<th>Extraction Conditions</th>
<th>Apparent M&lt;sub&gt;a&lt;/sub&gt; (kDa)</th>
<th>Real M&lt;sub&gt;a&lt;/sub&gt; (kDa)</th>
<th>Protein (mass %)</th>
<th>Polysacc. (mass %)</th>
<th>Mannose (molar %)</th>
<th>Glucose (molar %)</th>
<th>Other Sugars (molar %)</th>
<th>Phosphorus (mass %)</th>
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<td>-</td>
<td>-</td>
<td>21</td>
<td>79</td>
<td>97.5</td>
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<td>60-23</td>
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<td>96</td>
<td>78</td>
<td>13</td>
<td>9</td>
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<td>(Waters et al., 1994)</td>
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<td>-</td>
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<td>(Lubbers et al., 1993)</td>
<td>Purified/Fractionated after AF</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>90</td>
<td>87.7</td>
<td>12.3</td>
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<td>-</td>
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<tr>
<td></td>
<td>(Dufrechou et al., 2015)</td>
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<td>62</td>
<td>-</td>
<td>1.6</td>
<td>NS</td>
<td>88.8</td>
<td>2.6</td>
<td>8.5</td>
<td>-</td>
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<tr>
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<td>(Chalier et al., 2007)</td>
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<td>NS</td>
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<td>13</td>
<td>87</td>
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<td>91.1</td>
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<td>(Vernhet et al., 1996)</td>
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<td>NS</td>
<td>92.4</td>
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<td>1.7</td>
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<td>(Gonçalves et al., 2002)</td>
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<td>252-560</td>
<td>10.3</td>
<td>NS</td>
<td>100</td>
<td>ND</td>
<td>ND</td>
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<td>2.5</td>
<td>NS</td>
<td>87.5</td>
<td>2.6</td>
<td>9.8</td>
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<td>Physico-chemical treatments</td>
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<td>120°C/90min</td>
<td>-</td>
<td>-</td>
<td>35</td>
<td>63.5</td>
<td>97.6</td>
<td>1.1</td>
<td>1.3 (GlcNAc)</td>
<td>1.5</td>
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<tr>
<td></td>
<td>(Moine-Ledoux &amp; Dubourdieu, 1999)</td>
<td>120°C/90min</td>
<td>-</td>
<td>-</td>
<td>4.2</td>
<td>93.8</td>
<td>92</td>
<td>8</td>
<td>ND</td>
<td>-</td>
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<tr>
<td></td>
<td>(Núñez et al., 2006)</td>
<td>85°C/24h</td>
<td>-</td>
<td>-</td>
<td>31.3</td>
<td>66.1</td>
<td>61.3</td>
<td>4.9</td>
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<tr>
<td></td>
<td>(De Iseppi et al., 2019)</td>
<td>120°C/90min + 1min St</td>
<td>&gt;250</td>
<td>-</td>
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<td>92.1</td>
<td>84</td>
<td>16</td>
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<td>-</td>
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<td>90.9</td>
<td>80</td>
<td>10.9</td>
<td>ND</td>
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<td>150-250</td>
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<td>98.7</td>
<td>69.4</td>
<td>30.6</td>
<td>ND</td>
<td>-</td>
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<tr>
<td></td>
<td>(Moine-Ledoux &amp; Dubourdieu, 1999)</td>
<td>Glucanex®</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>83.2</td>
<td>100</td>
<td>ND</td>
<td>ND</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1: Literature data on the characterization of mannoproteins extracted by different methods. M<sub>a</sub> measured through SDS-PAGE or Pullulan Calibration. M<sub>a</sub> 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 10⁶ 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,
Megazyme, Ireland). This enzyme also contains a small residual Endo-\(\beta\)-1,6-glucanase activity but no proteolytic activity (Schiavone et al., 2014). The enzyme solution (2mL) was added to the cell dispersion and the hydrolysis took place at 40 °C under magnetic stirring for 17-18 hours. The reaction was stopped by enzyme inactivation at 70°C for 15 min. The suspension was centrifuged (15000 g, 15min) and the supernatant was recovered in pre-treated dialysis bags of 6-8 kDa size exclusion cut-off (Spectrum, Canada). Dialysis was performed in deionized water for 3 days with water changes three times a day. Dialyzed extracts were recovered and concentrated to a final volume of about 40 mL by evaporation at low temperature (40 °C) under vacuum.

2.1.3 Purification of the Mannoproteins from the Enzymatic Extracts

The purification of the enzymatic extracts was performed through Anion Exchange Chromatography assisted by an NGC Chromatographic System (BioRad, USA) equipped with both diode array and conductivity detectors. Concentrated enzymatic extracts were diluted with a 30 mM citrate-phosphate buffer (McIlvaine, 1921) at pH 3.5 until the target pH (3.5-3.6) was reached. This buffer was prepared by mixing 69.65 mL of citric acid 0.1M and 30.35 mL of Na$_2$HPO$_4$ 0.2M before being 10-fold diluted. The solution was loaded in a prepacked HiPrep Q Sepharose XL 20 mL column (GE Healthcare, USA) equilibrated with the same buffer (eluent A). An unbound fraction was eluted with the equilibration buffer while a bound fraction (mainly proteins) was recovered through a linear ionic strength gradient: from 100 % eluent A to 100 % eluent B (eluent A + NaCl 2 M) in 15 min. The unbound fraction, corresponding to the whole mannoprotein content of the enzymatic extracts, was then further purified by ultrafiltration and successive diafiltrations (up to a dilution factor 1000) to remove organic acids and salts. Ultrafiltration was performed on a 30 kDa cut-off membrane composed of regenerated cellulose from Millipore (Burlington, USA) using an Amicon® device (Burlington, USA) under the pressure of 1.5 bar and stirring. The retentates (mannoprotein pools) were recovered, filtrated
10 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 cm²; Showa Denkko, Japan) coupled with an OH-pak KB-800P guard column. 50μL of the samples were eluted with a 0.45 μm filtered LiNO₃ 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
replaced by a Quasi-Elastic Light Scattering detector (QELS) (DAWN–Heleos II from Wyatt, CA, USA), an on-line differential viscometer (VISCOSTAR II, Wyatt, CA, USA), an UV detector (280 nm) (SPD-20A, Shimadzu, Japan) and a differential refractometer (666 nm, Optilab T-Rex, Wyatt, Santa Barbara, CA, USA). Pools were dissolved in Milli-Q water at 4g.L\(^{-1}\) and centrifuged at 18 000 g/15 min before being eluted with a filtered (0.1 \(\mu\)m filter, mixed cellulose ester, Millipore) solution (0.1 M LiNO\(_3\) + 0.02% NaN\(_3\)) at a flow rate of 1 mL.min\(^{-1}\) and 40 °C. Data were analyzed using the Astra 6.1.2 software package. An average refractive index increment value (dn/dc) of 0.150 mL.g\(^{-1}\) was used for the polysaccharide moiety, n being the refractive index of the polysaccharide solution and c the polysaccharide concentration in mass. The protein modifier method within the software was used to estimate the respective molar masses of the protein and polysaccharide parts throughout the elution profiles. To this end, the mass extinction coefficient of the protein part was determined from UV absorbency measurements at 280 nm, performed with the whole mannoprotein pools. An average value of 1.4 mL.mg\(^{-1}\).cm\(^{-1}\) was determined and considered.

### 2.2.2 Neutral sugar analysis.

The neutral glycosyl-residues composition of the enzymatic extracts and mannoprotein pools was determined by gas chromatography after polysaccharides hydrolysis with Trifluoroacetic acid (Albersheim, Nevins, English, & Karr, 1967) and neutral sugar conversion in their alditol acetate derivatives (Blakeney, Harris, Henry, & Stone, 1983). Inositol and allose were added to the samples, after hydrolysis and before reduction and acetylation, as internal standards for quantification (Doco, Quellec, Moutounet, & Pellerin, 1999). The monosaccharides were identified through their retention time. GC-FID (Flame Ionization Detector) was performed by a SHIMADZU GC-2010-Plus gas chromatography system using a fused silica capillary column DB-225 (30m x 0.25 \(\mu\)m x 0.25 mm ID) (Agilent J&W, Santa 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 \( \mu \text{L} \) 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 \( \text{NaBD}_4 \), 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 \( \mu \text{L} \) 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.
<table>
<thead>
<tr>
<th></th>
<th>YC-Com</th>
<th>YC-WT</th>
<th>YC-Mnn4</th>
<th>YC-Mnn2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total biomass used</td>
<td>11</td>
<td>12</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>A Yield of enzymatic extraction (mg EE/g YC)</td>
<td>119.2</td>
<td>157.7</td>
<td>124.5</td>
<td>122.4</td>
</tr>
<tr>
<td>Protein (mass %)</td>
<td>21.2 ± 3.5</td>
<td>24.8 ± 1.4</td>
<td>25.1 ± 2.0</td>
<td>26.9 ± 8.0</td>
</tr>
<tr>
<td>Polysaccharide (mass %)</td>
<td>62.9 ± 0.6</td>
<td>60.9 ± 7.3</td>
<td>65.9 ± 2.9</td>
<td>60.9 ± 8.9</td>
</tr>
<tr>
<td>Mannose (molar %)</td>
<td>80.8 ± 2.4</td>
<td>71.1 ± 4.6</td>
<td>71.2 ± 0.9</td>
<td>52.6 ± 1.2</td>
</tr>
<tr>
<td>Glucose (molar %)</td>
<td>17.4 ± 2.0</td>
<td>27.2 ± 4.5</td>
<td>27.7 ± 0.8</td>
<td>46.1 ± 1.4</td>
</tr>
<tr>
<td>B Yield of MP production (mg MP/g YC)</td>
<td>101.2</td>
<td>116.3</td>
<td>105.9</td>
<td>103.1</td>
</tr>
<tr>
<td>Protein (mass %)</td>
<td>16.0 ± 1.1</td>
<td>18.0 ± 2.2</td>
<td>20.0 ± 1.3</td>
<td>20.0 ± 5.1</td>
</tr>
<tr>
<td>Polysaccharide (mass %)</td>
<td>59.5 ± 3.3</td>
<td>65.1 ± 0.7</td>
<td>62.2 ± 0.4</td>
<td>60.6 ± 4.2</td>
</tr>
<tr>
<td>Mannose (molar %)</td>
<td>82.3 ± 0.2</td>
<td>71.5 ± 0.5</td>
<td>72.3 ± 0.2</td>
<td>56.5 ± 0.2</td>
</tr>
<tr>
<td>Glucose (molar %)</td>
<td>16.2 ± 0.1</td>
<td>26.7 ± 0.6</td>
<td>26.2 ± 0.1</td>
<td>42.2 ± 0.1</td>
</tr>
<tr>
<td>Ratio Mannose/Glucose</td>
<td>5.1</td>
<td>2.7</td>
<td>2.8</td>
<td>1.3</td>
</tr>
<tr>
<td>C Yield of MP production (mg MP/g YC)</td>
<td>59.3</td>
<td>86.4</td>
<td>79.4</td>
<td>69.1</td>
</tr>
<tr>
<td>Protein (mass %)</td>
<td>4.3 ± 0.3</td>
<td>4.0 ± 0.3</td>
<td>3.6 ± 0.3</td>
<td>5.4 ± 0.4 a</td>
</tr>
<tr>
<td>Polysaccharide (mass %)</td>
<td>85.0 ± 2.3</td>
<td>77.4 ± 1.8</td>
<td>82.6 ± 2.3 ab</td>
<td>82.3 ± 2.2 ab</td>
</tr>
<tr>
<td>Mannose (molar %)</td>
<td>91.2 ± 0.0</td>
<td>83.7 ± 0.8</td>
<td>82.4 ± 1.4 b</td>
<td>67.3 ± 0.2 c</td>
</tr>
<tr>
<td>Glucose (molar %)</td>
<td>6.8 ± 0.2</td>
<td>14.8 ± 0.3 c</td>
<td>15.8 ± 0.6 b</td>
<td>30.6 ± 0.3 a</td>
</tr>
<tr>
<td>Ratio Mannose/Glucose</td>
<td>13.5</td>
<td>5.6</td>
<td>5.2</td>
<td>2.2</td>
</tr>
</tbody>
</table>

**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.

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

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.

<table>
<thead>
<tr>
<th>Glycosyl Residue</th>
<th>MP-Com</th>
<th>MP-WT</th>
<th>MP-Mnn4</th>
<th>MP-Mnn2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-Glucose</td>
<td>Glc-(1→</td>
<td>0.7</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>2,3,4-Glucose</td>
<td>→6)-Glc-(1→</td>
<td>2.2</td>
<td>5.7</td>
<td>6.4</td>
</tr>
<tr>
<td>2,4-Glucose</td>
<td>→3,6)-Glc-(1→</td>
<td>-</td>
<td>-</td>
<td>1.6</td>
</tr>
<tr>
<td>Total Glucose (% molar)</td>
<td>2.8</td>
<td>7.3</td>
<td>9.5</td>
<td>28.5</td>
</tr>
<tr>
<td>2,3,4,6-Manose</td>
<td>Man-(1→</td>
<td>29.7 (30.6)</td>
<td>30.6 (33.0)</td>
<td>29.5 (32.6)</td>
</tr>
<tr>
<td>2,3,4-Manose</td>
<td>→6)-Man-(1→</td>
<td>1.2</td>
<td>0.7 (0.8)</td>
<td>0.8 (0.9)</td>
</tr>
<tr>
<td>2,4,6-Manose</td>
<td>→3)-Man-(1→</td>
<td>18.9</td>
<td>11.4 (12.3)</td>
<td>10.3 (11.4)</td>
</tr>
<tr>
<td>3,4,6-Manose</td>
<td>→2)-Man-(1→</td>
<td>21.3</td>
<td>23.5 (25.4)</td>
<td>22.8 (25.2)</td>
</tr>
<tr>
<td>2,4-Manose</td>
<td>→3,6)-Man-(1→</td>
<td>0.6</td>
<td>0.4 (0.4)</td>
<td>0.6 (0.7)</td>
</tr>
<tr>
<td>3,4-Manose</td>
<td>→2,6)-Man-(1→</td>
<td>25.2</td>
<td>25.8 (27.8)</td>
<td>26.5 (29.3)</td>
</tr>
<tr>
<td>3-Manose</td>
<td>→2,4,6)-Man-(1→</td>
<td>0.3</td>
<td>0.2 (0.2)</td>
<td>0.3 (0.3)</td>
</tr>
<tr>
<td>Total Mannose (% molar)</td>
<td>97.2 (100.0)</td>
<td>92.7 (100.0)</td>
<td>90.5 (100.0)</td>
<td>71.5 (100.0)</td>
</tr>
<tr>
<td>Branching Degree (DB) (%)</td>
<td>56</td>
<td>60</td>
<td>62</td>
<td>13</td>
</tr>
</tbody>
</table>

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

**Table 5:** Average molecular parameters of the two main populations observed in the HPSEC-MALS profiles of the four mammoprotein pools

(MP-Com, MP-WT, MP-Mnn2, and MP-Mnn4). Mw: Molecular weight in mass, Mn: Molecular weight in number, Pdl: Polydispersity Index, R_g: Radius of Gyration, R_h: Hydrodynamic radius, [η]: intrinsic viscosity.
The predominant populations in the four mannoprotein pools (populations 2) had average 
$M_w$ ranging between $3.4 \times 10^5$ (MP-Com) and $8 \times 10^5$ (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 $\beta$-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).
The Intrinsic Viscosity $[\eta]$, radius of gyration ($R_g$), hydrodynamic radius ($R_h$), and the $\rho$ parameter ($\rho = R_g/R_h$) 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, $[\eta]$, $R_g$, and $R_h$ are related to the $M_w$ through a simple power law:

$$[\eta] = K_\alpha M_w^\alpha$$
\[ R_g = K_g M_w^{vG} \]  
\[ R_h = K_h M_w^{vH} \]

where \( \alpha, vG, \) and \( vH \) are the corresponding hydrodynamic coefficients and \( K_\alpha, K_g, \) and \( K_h \) the corresponding constants. The values of \( \alpha, vG, \) and \( vH \) provide some insights into the shape, anisotropy, and solvation of the macromolecules in the medium (good solvent or not) (Burchard, 1999). The \( \rho \) 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 \( \rho \) values.

The Mark-Howink-Sakurada plots ([\( \eta \)] = 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 \( \alpha \) coefficient) highlighted structural differences between these two populations (Ioan et al., 2000; Lopez-Torrez, Nigen, Williams, Doco, & Sanchez, 2015). \( \alpha \) values go from 0 (corresponding to a sphere-shape population) to 1.8 (rod-like shape). Flexible polymers usually have intermediate \( \alpha \) values (0.5-0.8), dependent on the interactions between the macromolecules and the solvent (Burchard, 1999). \( \alpha \) evolves inversely to their degree of branching and values below 0.5 indicate a hyperbranched polymer structure. Also, \( \alpha \) decreases as the M_w increases for macromolecules with a branching degree higher than 0.5. The \( \alpha \) 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. \( \alpha \) 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 \( \alpha \) was observed between population 2 and population 1 for all MPs, the \([\eta]\) becoming independent on Mw (\( \alpha \sim 0 \)) 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 [η] is mainly governed by the degree of branching
of the protein moiety.

For the same Mw, differences were observed in the Rg and Rh values of the
mannoproteins (Figure 5B and C). Both the νG (around 0.3) values obtained for the two
populations of MP-Com (Red) were consistent with a sphere-like conformation (Ioan, 1999).
The νH values around 0.4 found on the whole Mw 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 νH values determined within the “low” Mw 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, νH 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 νG 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 $\alpha$ values between the two populations of these mannoproteins and with the
decrease in $\nu H$: 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.
The $\rho$ parameter found for MP-Com and the high molar mass population in the three other MP pools varied with the $M_w$ between 1.1 and 0.9, which is consistent with dendrimeric (0.977) or regular star (1.079-1.33) structures (Supplementary Data, Table 2), and is within the range of $\rho$ found for other branched polysaccharides such as amyloses, dextrans, glycogens, and some fractions of Acacia Senegal and Acacia Seyal gums (Ioan et al., 1999; D Renard et al., 2014; Rolland-Sabaté et al., 2008). By contrast, for population 2 of MP-WT/Mnn4/Mnn2, a strong decrease of $\rho$ from 2 to 1.1 was observed when the $M_w$ increased. Theoretical $\rho$-values above 1.3 are usually related to linear random coil structures, which do not correspond to mannoproteins as confirmed by the power law coefficients obtained from MHS, $R_g x M_w$, and $R_h x M_w$ plots and the glycosyl-linkage composition analysis. In this specific case, this is related to the molar mass dependence of $R_g$ and $R_h$, attributed to an increase in branching density by carbohydrate side chains with the $M_w$ and/or increasing side chain length.

Overall, these results clearly show structural differences between MP-Com, MP-WT/Mnn4, and MP Mnn2, and differences between the “low” and “high” molar mass populations within the four pools. Any comparison with other biopolymers was limited by the fact that there is no consistent literature data on $\alpha$, $\nu_G$ and $\nu_H$ coefficients of mannoproteins, and that their composition as a natural copolymer is quite specific.

4 Conclusion

The extraction and purification methods applied in this study made it possible to obtain pools of mannoproteins from 4 yeast strains: a laboratory wild-type strain (BY4742), two of its mutants ($\Delta$Mnn4 and $\Delta$Mnn2, in which genetic deletions affect the polysaccharide moiety), and a commercial enological strain (LMD47) as control. The differences in the molecular characteristics of mannoproteins caused by genetic deletions ($\Delta$Mnn2 and $\Delta$Mnn4) on the strain BY4742 were confirmed and differences were also observed between the laboratory and the enological strains.
The composition of the protein and polysaccharide moieties of MPs showed that the protein moiety between the four MPs pools are similar and that the main difference comes from the polysaccharide part. As expected, differences from the normal polysaccharide structure of MP-Mnn2 and MP-Mnn4 when compared to MP-WT altered their net charge balance – usually ruled by both the protein and polysaccharide moieties – and the molecular conformation of MP-Mnn2. These characterizations also evidenced differences in structure, composition, and properties between MP-Com and MP-WT. Moreover, the comparison of the mannoproteins obtained in this work with the literature data, in terms of net charge, apparent and/or real molecular weight distributions, and protein content confirmed that the extraction method developed has little effect on the native structure of mannoproteins. In fact, mannoproteins purified from wine or extracted by the use of enzymatic cocktails (such as Zymolase® or Glucanex®) are also submitted to proteolytic and/or mannolytic activities and might not be representative of their structure in the yeast cell wall. The analysis of the static and dynamic parameters provided by HPSEC-MALLS revealed very specific conformational properties of mannoproteins related to their nature of highly branched natural copolymers with two branching levels: a protein more or less branched by irregular polysaccharides chains, which in turn are also more or less branched. However, any comparison with other sources of mannoproteins was limited by the fact that there is no literature data on $\alpha$, $\nu_G$ and $\nu_H$ coefficients for these macromolecules.

The four mannoprotein pools obtained and characterized will be used to study how the structure of the polysaccharide moiety, which affects the structure and conformation of the macromolecule, and mannosyl-phosphate groups, which carry a negative charge at wine pH, impact their interactions with wine constituents and their functional properties in enology.


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