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Analytical Methods

A new methodology to determine cell wall mannoprotein content and release in wine yeasts

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ABSTRACT

Representing around 40% of the cell wall dry weight, mannoproteins are complex macromolecules structurally composed of polymers of sugar, 98% being mannose, covalently linked to peptides. Along the last two decades, these compounds have gained ground as very relevant molecules in the field of winemaking, mainly due to their positive contributions in the development of appreciated organoleptic features and to their contribution in the chemical stabilization of wine. Several methodologies have been recently proposed to achieve the quantification of these compounds. However, these methodologies are laborious, time consuming and do not allow a global quantification of these macromolecules. In this paper, an easy, reliable and fast forward methodology for the quantification of mannoproteins in model must is proposed and evaluated. Its application in the quantification of mannoproteins content in yeast cell wall is also demonstrated.

1. Introduction

Over the last two decades, yeast mannoproteins have become a hot topic in the field of winemaking. Numerous studies have clearly proven the multiple positive contributions of these molecules to the overall vinification process (Doco, Vuchot, Cheynier, & Moutounet, 2003; Dufour & Bayonove, 1999; Dupin et al., 2000; Guadalupe & Ayestarán, 2008). Amongst their most outstanding oenological features, mannoproteins contribute to the chemical stabilization of wine by preventing crystallization of tartrate salts (Feuillat, Charpentier, & Nguyen van Long, 1998; Gerbaud et al., 1996) and protecting against protein haze (Dupin et al., 2000; Gonzalez-Ramos, Cebollero, & Gonzalez, 2008; Gonzalez-Ramos & Gonzalez, 2006; Gonzalez-Ramos, Quirós, & Gonzalez, 2009; Waters, Pellerin, & Brillouet, 1994). These highly glycosylated proteins stimulate growth of lactic-acid bacteria in wine environments and thus the development of malolactic fermentation (Guilloux-Benatier & Chassagne, 2003) and allow a reduction in the concentration of some undesired compounds such as ochratoxin A (Ringot et al., 2005). Furthermore, mannoproteins have a relevant impact on the sensorial properties of wine as they retain aroma compounds (Lubbers, Voilley, Feuillat, & Charpentier, 1994), reduce astringency, improve the foaming properties of sparkling wines (Núñez, Carrascosa, Gonzalez, Polo, & Martínez-Rodríguez, 2006) and increase the body, sweetness, roundness and mouthfeel of the final product (Guadalupe, Palacios, & Ayestarán, 2007; Vidal et al., 2004).

Mannoproteins are mainly located in the outermost layer of the yeast cell wall, where they act as structural components and are partially responsible for its permeability (Klis, Mol, Hellingwerf, & Brul, 2002). On average, 30% of their structure corresponds to the protein fraction, while the remaining 70% corresponds to sugar residues, 98% of which are mannose. The release of yeast mannoproteins during winemaking mainly takes place when alcoholic fermentation has come to an end. At that point, cell viability drastically decreases and cell death leads to the release of its constituents in a phenomenon known as autolysis.

Although little is known about the genetic determinants involved in the secretion of mannoproteins during wine fermentations, several recent papers have cast light onto this issue (Gonzalez-Ramos & Gonzalez, 2006; Gonzalez-Ramos et al., 2008, 2009). The results presented in these works have led to the development of a new methodology to obtain wine yeast strains overproducing mannoproteins (Quirós, Gonzalez-Ramos, Tabera, & Gonzalez, 2010). While working on this topic, the need to establish a standardised and reliable methodology to perform the quantification of these molecules became obvious. Some currently available methodologies are either unspecific (Segarra, Lao, López-Tamames, & de la Torre-Boronat, 1995) or complicated

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and laborious and do not allow a clear overall quantification of the release of mannoproteins (Palomero et al., 2009).

In the present paper, a simple and reliable methodology to quantify the release of mannoproteins by yeast strains in a synthetic medium and in model must and to determine their concentration in the cell wall is proposed and evaluated. Correlation studies to predict the potential behaviour of yeast strains in mannoproteins release in wine fermentations have also been investigated.

2. Materials and methods

2.1. Strains and culture conditions

Eight different *Saccharomyces cerevisiae* strains and one hybrid *S. cerevisiae* × *Saccharomyces kudriavzevii* were used in this work. Within the first set of strains, BM45, D254, EC1118, T73 and VRB are wine yeast strains commercialised by Lallemand Inc. (Ontario, Canada). IFI87 and IFI475 are non-commercial winemaking strains obtained from the Instituto de Fermentaciones Industriales culture collection (CSIC) while BY4743 is a reference laboratory strain obtained from the Euroscarf consortium. Finally, W27, the only natural *S. cerevisiae* × *S. kudriavzevii* hybrid used (González, Barrio, Gafner, & Querol, 2006) was originally isolated in Wädenswil, Switzerland, and is also commercialised by Lallemand Inc. Strains were routinely maintained on YPD plates, containing 2% glucose, 2% peptone, 1% yeast extract and 2% agar.

The release of mannoproteins and polysaccharides and the mannoprotein content in the cell wall of the selected strains was studied in two different media. Firstly, fermentations were performed in 50 ml of GCY, a synthetic medium containing 2% glucose (Sigma–Aldrich, St. Loius, MO), 2% Bacto™ Casamino Acids (BD, Sparks, USA) and 0.67% Difco™ Yeast Nitrogen Base (BD) during 24 h at 30 °C and 150 rpm shaking. In a second step, industrial strains were used in order to perform fermentations in a synthetic must (pH 3.5) containing 10% glucose, 10% fructose, 0.6% citric acid, 0.6% malic acid (all of them from Sigma–Aldrich), 0.17% YNB without aminoacids and ammonium sulphate (Difco), 306 mg/l NH_4Cl (Panreac, Barcelona, Spain) and 2/3 of the amount of aminoacids used in the control synthetic grape must (CNC) described by Beltran, Novo, Rozès, Mas, and Guillamón (2004), corresponding to 200 mg/l of yeast assimilable nitrogen. In this case, fermentations were performed in 100 ml borosilicate glass bottles (Schott AG, Mainz, Germany) containing 50 ml of medium. Bottles were capped with Müller valves filled with vaseline oil and incubated at 25 °C without shaking. Fermentation time courses were monitored by determining the production of CO_2 expressed as weight loss until weight was constant.

Inocula were grown in YPD broth o/n, washed twice in sterile distilled water and inoculated at a final concentration of 10^6 cells/ml (approximately 0.1 units $OD_{600\ nm}$).

2.2. Quantification of total mannoproteins and polysaccharides released during fermentation

When all fermentations were finished, i.e. sugar was depleted, cultures were centrifuged at 10,000 rpm for 5 min and 3 ml of each supernatant was gel filtered through 30 × 10 mm Econo-Pac® 10 DG disposable chromatography columns (Bio-Rad Laboratories, Hercules, CA) and eluted with 4 ml distilled water in order to isolate the non-retained macromolecular fraction. For samples corresponding to fermentations in synthetic must, 3 ml of the eluted fraction were filtered again using the same type of columns and eluted with 4 ml of distilled water. For samples corresponding to fermentations in GCY medium, this second filtration step was not necessary. Once the macromolecular fraction was obtained, it was subjected to a double analysis. On one side, three aliquots of 200 μl were subjected to the phenol–sulphuric acid method described by Segarra et al. (1995). Absorbance at 490 nm was determined using a UV-1800 Shimadzu spectrophotometer (Shimadzu Corp., Kyoto, Japan). In parallel, two aliquots of 2 ml were concentrated in 2 ml screw-capped microtubes (Sarstedt, Nümbrecht, Germany) using a miVac DNA centrifugal concentrator (Genevac Ltd., Suffolk, UK) at 60 °C until complete evaporation. Resulting pellets were carefully resuspended in 100 μl of 1 M H_2SO_4. Tubes were tightly capped and placed in an oven at 100 °C for 4 h to undergo acid hydrolysis. After this treatment, tubes were briefly spun down, 10-fold diluted using 900 μl of miliQ water, filtered through 0.45 μm pore size nylon filters (Scharlab, Sentmenat, Spain) and subjected to HPLC analysis for quantification of the glucose and mannose released during hydrolysis. For the preparation of a standard curve, serial aqueous dilutions of commercial mannan from *S. cerevisiae* (Sigma–Aldrich) containing 10 different concentrations, ranging from 250 to 25 mg/l, were prepared and subjected to the double hydrolysis described above.

2.3. Determination of the relative mannoprotein content of the yeast cell wall

In order to obtain an indicative value of the mannoprotein content in the yeast cell wall, two samples of 3 ml of each of the fermentations performed in GCY and synthetic must were centrifuged, supernatant discarded and cells washed twice with 5 ml sterile distilled water. Pellets were then subjected to acid hydrolysis following the protocol described above (Section 2.2). The glucose and mannose content was determined by HPLC and the mannose:glucose ratio of the cell wall calculated.

2.4. Stability of the hydrolysed samples

In order to check the stability of the hydrolysed samples, a cell wall hydrolysis corresponding to strain EC1118 was prepared as mentioned above, aliquoted in eppendorf tubes and stored at −20, 4 and 28 °C for 5 and 15 days. After that time, samples were analysed by HPLC and the mannose:glucose ratio obtained compared to the one observed for the same sample analysed just after the hydrolysis had been performed.

2.5. Method reproducibility and repeatability

In order to characterise the reproducibility and repeatability of the methodology proposed and compare it to those obtained for the phenol–sulphuric method used as reference, 15 ml of a macromolecular fraction from two independent cultures of the strains EC1118 and T73 in synthetic must were obtained by column filtration as described above. Three independent manipulators performed a double hydrolysis of the concentrated macromolecular fraction followed by HPLC analysis and a triple determination of the total polysaccharide content using the phenol–sulphuric method in two different types of commercial 2 ml tubes in order to analyse the possible effect of the shape and/or the plastic material in the determination.

2.6. HPLC analysis of hydrolysates, musts and wines

To determine the concentration of glucose and mannose resulting from the acid hydrolysis of both the macromolecular fractions and yeast cell walls, samples were filtered through 0.45 μm pore size nylon filters (Symta, Madrid, Spain) and injected in duplicate into a Surveyor Plus chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector (Surveyor